REGULATION OF UTERINE ENDOMETRIAL GENE EXPRESSION AND GROWTH BY THE SP/KRUPPEL-LIKE FAMILY MEMBER, BASIC TRANSCRIPTION ELEMENT BINDING PROTEIN 1

By

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This dissertation is dedicated to my parents with deepest appreciation and to my husband with love and thanks from my heart.

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REGULATION OF UTERINE ENDOMETRIAL GENE EXPRESSION AND GROWTH BY THE SP/KRUPPEL-LIKE FAMILY MEMBER, BASIC TRANSCRIPTION ELEMENT BINDING PROTEIN 1

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Basic transcription element binding protein (BTEB1) is a transcription factor that regulates gene expression through interactions with regulatory DNA elements of target genes. This dissertation examined the molecular mechanisms underlying pregnancy-associated gene expression, and the consequent effects on uterine endometrial epithelial cell proliferation and differentiation mediated by BTEB1.

Using the pregnant pig as an animal model, mRNA and protein levels of BTEB1 and its well-known Krüppel-like family member, Sp1, were examined in primary cultures of uterine endometrial glandular epithelial cells treated with estrogen and progesterone. The results showed that while the protein abundance of both BTEB1 and Sp1 were not affected by either steroid hormone, their mRNA levels were modestly increased by estrogen in glandular epithelial cells isolated from uterine endometrium of animals with lower estrogen background.

Functional association of liganded progesterone receptor and BTEB1 on the regulation of pregnancy-associated gene expression also was investigated. The results showed that BTEB1 increased the threshold sensitivity for transcriptional activation of the pregnancy-associated gene uteroferrin to progesterone in the presence of progesterone receptor isoform B, but not isoform A, in transiently transfected human endometrial adeno-carcinoma Hec-1-A cells. Further, this effect was dependent on target gene promoter context. Another Sp/Krüppel-like family member, BTEB3, had a similar effect as BTEB1 on progesterone receptor B-mediated transactivation indicating common mechanisms utilized by Sp/Krüppel-like family members and nuclear receptors on the regulation of uterine gene expression.

It was also demonstrated that BTEB1 regulates uterine endometrial epithelial cell proliferation through its transactivation of the pregnancy-associated gene encoding secretory leukocyte protease inhibitor (SLPI). Using "*in vitro* SLPI knock out" cells, novel pathways involving SLPI induction of uterine endometrial epithelial cell proliferation were demonstrated. SLPI increased cyclin D1 gene expression, and decreased the expression of the growth inhibitors transforming growth factor-β1, lysyl oxidase, and insulin-like growth factor-binding protein-3 genes.

The collective results from these studies demonstrate that BTEB1 protein is an important pregnancy-associated transcription factor that affects uterine endometrial cell differentiation and proliferation to support successful pregnancy.

CHAPTER 1
INTRODUCTION

Implantation of the embryo into the receptive endometrium is a complex process. This is a critical step in the establishment of pregnancy, and its success depends on the highly coordinated processes of the differentiation of uterine endometrium, development of the embryo, and the correct maternal-embryo interactions. The process of implantation and events thereafter are tightly regulated by a myriad of molecules including growth factors, proteases, protease inhibitors, and cytokines, which are specifically expressed during this time period. The genes encoding these molecules are found to be pregnancy-associated, and their specific spatial and temporal modes of expression within the uterine environment are tightly regulated not only by the pregnancy hormones, estrogen and progesterone bound to their respective nuclear receptors, but by other molecules as well.

Progesterone exerts a critical role in preparation of the uterine endometrium for embryo implantation. Progesterone, in concert with estrogen and uterine-associated growth factors, coordinates cellular proliferation, differentiation, and apoptosis in a temporal- and spatial-specific manner, by binding to its nuclear receptor (Tsai and O'Malley, 1994). Two progesterone receptor (PR) isoforms (namely PR-A and PR-B) were identified to mediate the effects of progesterone (Kastner et al., 1990). Both are expressed from the same gene but from two alternative promoters; PR-B has extra 164 amino acids in its N-terminus relative to PR-A. This difference in primary structures

between the two PRs underlies their distinct functions. PR-B usually functions as a ligand-dependent transactivator of progesterone-responsive genes in a specific cell-type and target gene promoter context; in contrast, PR-A acts as a ligand-dependent transcriptional repressor of PR-B as well as of other steroid hormone receptors (Kastner et al., 1990; Vegeto et al., 1993; McDonnell, 1995; Giangrande et al., 1997; Hovland et al., 1998). A recent study indicated that the functional difference between PR isoforms can be partially related to the distinct conformations achieved by each isoform in the presence of ligand, resulting in the isoform's differential ability to bind to transcriptional co-activators or co-repressors of transcription (Giangrande et al., 2000).

Sp1 and BTEB1 are potential partners of PR in regulating uterine endometrial gene expression during pregnancy. They are members of the Sp/Krüppel-like family of transcription factors, which bind GC-rich motifs widely distributed within gene promoters through their C_2H_2 zinc finger DNA-binding domain, resulting in distinct activation or repression of transcriptional activities. Sp1 protein is considered to be more ubiquitously distributed in mammalian tissues than BTEB1, although their mRNAs are expressed in almost all tissues (Imataka et al., 1992; Imataka et al., 1994). Imataka et al. (1994) suggested that the post-transcriptional regulation of BTEB1 gene may be mediated by the 10 AUGs located in the long 5'UTR of the BTEB1 mRNA. In addition, a highly GC-rich region in the 5'UTR, together with the first 95 bases of the BTEB1 coding region, is predicted to form a stable stem-loop structure (Yasumoto, 1993), which could form a secondary structure and impede the 40S preinitiation complex to move along the mRNA to repress translation. In pregnant porcine uterine endometrium, BTEB1 protein is present in glandular and luminal epithelial cells but not in stromal cells,

although its mRNA exists in all three major endometrial cell types (Wang et al., 1997). To investigate the role of BTEB1 in pregnancy, studies were conducted to examine the functional relationship between BTEB1 and the steroid hormones, estrogen and progesterone. A recent study has shown that progesterone regulates Tissue Factor gene in human endometrial stromal cells through the induction of Sp1 and inhibition of Sp3 gene expression, respectively (Krikun et al., 2000). Another study indicated that BTEB1 gene expression was induced by thyroid hormone in Neuro-2A cells, consistent with the observed increase in the levels of BTEB1 mRNA in developing rat brain (Denver et al., 1999). These interesting observations led to the hypothesis that BTEB1 and Sp1 gene expression may be regulated by estrogen and progesterone in uterine endometrium during pregnancy.

Besides the regulation of this gene expression by steroid hormones, Sp/Krüppel-like family members act as potential partners of PR in regulating gene expression during the pregnancy. A recent study described Sp1 as a PR-interacting partner (Kadonaga et al., 1987; Turner and Crossley, 1999). Sp1 was shown to mediate the transactivation of cyclin-dependent kinase inhibitor p21 gene promoter by forming a functional multi-protein complex with PR/P and the transcriptional co-activator CBP/p300 (Owen et al., 1998). Sp1 has also been implicated in the regulation of estrogen-, retinoic acid- and androgen-responsive genes via the formation of similar complexes (Qin et al., 1999; Husmann et al., 2000; Petz and Nardulli, 2000). Although at least 20 Sp/Krüppel-like family members have now been identified, no evidence has yet been provided to indicate that with the exception of Sp1, other family members, which display more spatial-, temporal- and developmental-specificity than Sp1, can mediate PR-dependent gene

regulation in target cells. In this dissertation, studies were conducted to a) investigate the effects of BTEB1 and its closely related family member, BTEB3 on PR-dependent gene regulation, b) elucidate the possible mechanism underlying the interaction of BTEB1 with PR-A or PR-B, and c) describe a possible role for BTEB1 in regulating pregnancy-associated gene expression and in mediating uterine endometrial epithelial cell differentiation.

During embryo implantation and subsequent pregnancy events, the uterine endometrium undergoes dramatic morphological and biological changes including cell proliferation and apoptosis. BTEB1 has been shown to be important in rat brain development (Denver et al., 1999). Previous studies in this laboratory demonstrated that BTEB1 mediated human uterine endometrial adenocarcinoma cell proliferation through its transactivation of several growth-related genes, including cyclin D1, PCNA, and Cdk2 (Zhang et al., 2001). Interestingly, the gene encoding secretory leukocyte protease inhibitor (SLPI) was also found to be transactivated by BTEB1 in this study. SLPI is a 12 kDa molecule belonging to the serine protease inhibitor family. It has two homologous domains, with the amino-terminal domain representing the trypsin-binding and the carboxyl-terminal domain representing the elastase and cathepsin G binding regions, respectively. SLPI is primarily expressed in epithelial cells, especially secretory/glandular epithelial cells of numerous tissues, and functions as an anti-inflammatory, anti-bacterial, anti-fungal, and anti-viral (HIV) agent (Hiemstra et al., 1996; Tomee et al., 1997; Wahl et al., 1997; Lentsch et al., 1999; Song et al., 1999; Gipson et al., 1999). The higher uterine endometrial expression of SLPI during pregnancy in species with epitheliochorial rather than haemochorial type of implantation

suggests an important role for SLPI in maintaining an intact utero-placental interface (Farmer et al., 1990; Badinga et al., 1994). SLPI was further demonstrated to exhibit an ability to increase DNA synthesis in porcine uterine endometrial glandular epithelial cells (Badinga et al., 1999). The latter provides a novel role for SLPI as a growth factor in pregnancy endometrium. Studies from other laboratories also support this new function of SLPI. Indeed, SLPI has been shown to support the growth of human hematopoietic progenitor cells in serum-free medium *in vitro* (Goselink et al., 1996), and to increase the production of hepatocyte growth factor in human lung fibroblasts (Kikuchi et al., 2000). However, the mechanisms by which SLPI regulates epithelial cell proliferation remain unclear. Studies were conducted in the third part of this dissertation to determine if BTEB1 transactivates the SLPI gene promoter, and to further elucidate if the regulation by SLPI of growth-related genes represents one pathway by which BTEB1 induces uterine endometrial cell proliferation.

CHAPTER 2
REVIEW OF LITERATURE

Sp/Krüppel-like Family of Transcription Factors

Sp1

Introduction. Transcription factors, which directly participate in the regulation of gene expression by interacting with regulatory DNA elements in target genes, contribute to a large extent to the tissue-, cell-, and developmental-specific expression of eukaryotic genes. Sp1 represents the first identified and cloned protein of a family of transcription factors characterized by a highly conserved DNA-binding domain consisting of three zinc fingers. The ubiquitously-expressed Sp1 protein has been implicated in the activation of a very large number of genes and is thought to be involved in cellular processes such as cell cycle regulation, chromatin remodeling and the propagation of methylation-free islands (reviewed by Philipsen and Suske, 1999)

Background. Sp1 (named according to its original purification scheme that included Sephacryl and phosphocellulose columns) (Kadonaga et al., 1987) was first identified from Hela cell extracts as a promoter-specific transcription factor. Transcriptional activation by Sp1 required its direct binding to a 21 bp repeat sequence containing six GGGCGG motifs (GC-boxes) of the SV40 promoter (Dynan and Tjian, 1983). Sp1 was found to be expressed ubiquitously in mammalian cells as a transcriptional activator (Dynan and Tjian, 1985; McKnight and Tjian, 1986). A single gene encodes Sp1 (Kadonaga et al., 1987), and Sp1 mRNA has three sizes (8.2 kb, 5.2 kb

and about 3.5 kb), as determined by Northern blot analysis (Kadonaga et al., 1987; Imataka et al., 1992; Simmen et al., 2000). Purified Sp1 protein migrated on SDS-PAGE with apparent molecular weights of 95 kDa and 105 kDa respectively (Kadonaga and Tjian, 1986; Briggs et al., 1986), and the difference was subsequently shown to result from differential phosphorylation and glycosylation of a single polypeptide (Kadonaga et al., 1987; Jackson and Tjian, 1988).

Molecular structure of Sp1. The gene (Sp1-1) that encodes the transcription factor Sp1 has been located on the distal end of mouse chromosome 15 and human chromosome 12q (Saffer et al., 1990). It is a single copy gene linked to the mammary tumor virus integration site (Int-1) (Saffer et al., 1990) and is not linked to any other zinc finger protein genes that have been mapped in mouse. Sp1 acts in a binding site-dependent fashion, and the DNA binding activity of Sp1 is located to the C-terminal region that contains 168 amino acid residues that form three contiguous Zn (II) finger motifs (Kadonaga et al., 1987). Sp1 exhibits four distinct regions (termed regions A, B, C, and D), other than the zinc fingers, that are important for transcriptional activation (Courey and Tjian, 1988). Domains A and B in the N-terminal portion of Sp1 contain two glutamine-rich segments; These domains are largely devoid of charged residues in their primary sequences, and there is no correlation noted between charge, the length of a glutamine rich- segment and the transcriptional activity of Sp1. The serine/threonine-rich regions also located in Domain A and B of Sp1 are likely to be the sites for posttranslational modification, such as N-linked glycosylation (Marshall, 1972; Jackson and Tjian, 1988), and do not exhibit transcriptional activity. Domain C, which has only one-tenth the transcriptional activity of either of the two glutamine-rich domains, maps to

a segment of highly charged amino acid located just N-terminal to the zinc finger regions. Domain D, located at the extreme C-terminal end of the protein, does not function alone, but has a large effect on the full function of domains A, B, and C. This may be due to the ability of domain D to interact with the other activation domains, perhaps by helping to fold or position them for maximal activity (Courey and Tjian, 1988).

Mechanisms of actions. Sp1 recognizes the DNA sequence GGGCGG (GC box), found alone or in multiple numbers in many cellular and viral promoters. This binding allows Sp1 to activate gene transcription by its interactious with the TFIID complex through domains rich in glutamine residues (Kadonaga et al., 1987; Courey and Tjian, 1988; Pugh and Tjian 1990). Sp1 does not bind TBP (TATA-box-binding protein) but interacts at multiple sites with proteins of the TFIID complex. It is now quite clear that TAF110 specifically interacts with the glutamine-rich activation domains of Sp1 in both *Drosophila* cells and yeast (Hoey et al., 1993). Human $TAF_{II}55$ was also found to interact with the Sp1 DNA-binding domain (Chiang and Roeder, 1995). In some gene promoters that lack TATA and CAAT boxes but are rich in GC-boxes (a common feature of housekeeping gene promoters), examples of which include the human insulin receptor (HIR), the rat insulin-like growth factor-binding protein-2, and human adenosine deaminase, Sp1 is essential to their efficient transcription (Lee et al., 1992; Boisclair et al., 1993; Dusing and Wiginton, 1994). The function of Sp1 in TATA-less promoters may involve the recruiting of the TFIID complex through protein-protein interactions with Sp1, stabilizing components of the transcriptional machinery, and tethering TBP to the major transcription start site (Pugh and Tjian, 1990; Pugh and Tjian, 1991). Sp1 can bind to multiple GC-boxes, which results in a synergistic increase in activity much

greater than that obtained from its binding to individual GC-boxes (Pascal and Tjian, 1991). Electron microscopy studies showed that interactions occur between Sp1 molecules in promoters containing multiple GC-boxes that are widely separated (Su et al., 1991). Domain D of Sp1 was shown to be important for the intermolecular interactions between Sp1 molecules and mediates synergistic transcriptional activation (Pascal and Tjian, 1991).

Interactions with nuclear receptors to regulate gene expression. Sp1 is generally considered a global, housekeeping transcription factor whose expression is more constitutive relative to other family members (Imataka et al., 1992; Philipsen and Suske, 1999; Treichel et al., 2001). Some studies have shown that Sp1 regulates tissue- and development-dependent gene expression through its interactions with other transcription factors. In microglial cells, a functional synergism exists between Sp1 and the ovalbumin upstream promoter transcription factor (COUP-TF) leading to enhanced transcriptional activity of the HIV-1 long terminal repeat through the Sp1 element (Rohr et al., 1997). The physical interaction between Sp1 and the DNA-binding domain of COUP-TF was further demonstrated. Since COUP-TF can bind the TFIIB, a functional complex is formed between COUP-TF and Sp1 with the general transcription machinery. This mechanism suggested a general model for gene activation by the orphan receptor COUP-TF or other steroid/thyroid/retinoid receptors, in the presence of Sp1-binding sites. In another study, Sp1 was shown to mediate the transactivation of the cyclin-dependent kinase inhibitor p21 gene promoter, which lacks canonical promoter-response elements, by forming a functional multiprotein complex with PR/progesterone and the transcription activator CBP/p300 (Owen et al., 1998). A similar mechanism of regulation of the p21

gene expression by androgen through the formation of an androgen receptor-Sp1 complex was recently demonstrated (Lu et al., 2000). The direct (physical) interaction between Sp1 and estrogen receptor has also been demonstrated (Porter et al., 1997). Further, estrogen was shown to induce the transcription of insulin-like growth factor-binding protein-4 (Qin et al., 1999) and adenosine deaminase (Xie et al., 1999) genes in MCF-7 cells through the formation of an estrogen receptor-Sp1 complex; a site-specific GC-rich motif, rather than an estrogen- responsive element, was involved in the transactivation events. Another study demonstrated that an estrogen-induced change in the local chromatin structure of the rabbit uteroglobin gene during early pseudopregnancy facilitated the binding of Sp1 to its recognition motifs *in vivo* (Scholz et al., 1998). The same finding was noted in the regulation by estrogen of the human progesterone receptor A promoter activity (Petz and Nardulli, 2000).

Sp1 knock-out. The ubiquitously expressed Sp1 protein has been implicated in the activation of numerous genes and, thus, is thought to be involved in many biological processes. Studies on the Sp1 knockout mouse by Marin and colleagues (1997) indicated that Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. Sp1 null ES cells could be generated without any difficulty and these cells showed normal growth characteristics, maintained their methylation-free islands and could differentiate *in vitro* to form embryoid bodies with similar efficiencies as control ES cells. However, all Sp1 null embryos are severely retarded in growth and die after day 10 of development. They display a wide range of abnormalities at this stage. Some embryos appear as an undifferentiated mass of cells, while in others all the typical hallmarks of early embryogenesis are present, such as the developing heart, eyes, otic

vesicles, somites, erythroid cells and extraembryonic tissues. In Sp1 null embryos, the expression of many target genes, including cell cycle-regulated genes, is not affected. The phenotype of the Sp1 knockout suggests that Sp1 has an essential role in cellular survival during mouse development by regulating the expression of the gene encoding methyl-CpG-binding protein (MeCP2); the latter is proposed to be required for the maintenance of differentiated cells since mice null for this gene have remarkably similar phenotypes as those of Sp1 null mice (Tate et al., 1996).

BTEB1

Introduction. The transcription factor BTEB1 (basic transcription element binding protein 1), also known as KLF9, is a member of the Sp/Krüppel-like family. Similar to Sp1, BTEB1 exhibits a highly conserved C-terminal DNA-binding domain that consists of three zinc fingers that are found in the Drosophila protein, Krüppel. Both Sp1 and BTEB1 bind BTE, a GC box sequence in the P-4501A1 gene (Imataka et al., 1992), and their binding affinity to GC motifs is similar (Sogawa et al., 1993a). However, BTEB1 has distinct features that distinguish it from Sp1 and other family members. In particular, BTEB1 exhibits an exclusive transactivation domain, is translationally regulated distinct from those of other family members, and functions as an activator or a repressor of transcription.

Background. BTEB1 protein was first identified from a rat liver cDNA library by using synthetic BTE (a GC box sequence within the P-450A1 gene promoter) as a probe. The presence of BTEB1 mRNA (~5kb) was demonstrated in all rat tissues (Imataka et al., 1992). Subsequently, human BTEB was isolated from a human placental cDNA library and was found to be located on human chromosome 9 q13 by fluorescent *in situ*

hybridization and by DNA blot analysis using DNAs from hybrid cell clones containing a single human chromosome (Ohe et al., 1993). Both human and rat BTEB1 mRNAs encode a 244 amino acid polypeptide, whose primary sequence exhibits high sequence similarities (98% amino acid identity). The rat open reading frame is flanked by a long (> 500bp) stretch of GC-rich (73%) 5'-untranslated region (UTR) and 3'-UTR (>1.4 kb)(Imataka et al., 1992). The human BTEB1 mRNA carries a long 5' (> 1.2kb) and 3' (> 2.8 kb) UTR, respectively. The rat BTEB mRNA is ~5 kb in length, except in the testis where it is about 2 kb in length (Imataka et al., 1992).

Molecular structure and related functions of BTEB1. BTEB1 shares a similar DNA-binding region containing zinc finger domains with a Cys-Cys/His-His motif located at the C-terminal region with other Sp/KLF members. The similarity in primary sequence within the zinc-finger domains of the two transcription factors is 72% (58/81 amino acids) (Imataka et al., 1992). There is no difference in the recognition specificity or affinity between BTEB1 and Sp1 for the BTE sequence. Indeed, the dissociation constant of BTEB1 and the BTE oligonucleotide was comparable to or even lower than that of Sp1 and its recognition motif (Sogawa et al., 1993). The comparable affinities as well as specificities of BTEB1 and Sp1 for the GC-box suggest that BTEB1 competes with Sp1 for binding to the GC box present in target gene promoters. The lack of an additive effect of BTEB1 and Sp1 on the transactivation of the uteroferrin promoter, which contains five non-consensus GC-rich regions, supports the notion that BTEB and Sp1 bind to the DNA in exclusion of the other (Simmen et al., 2000). A short basic sequence (amino acid 135 to 144) immediately N-terminal to the zinc finger structure is involved in the DNA-binding activity or in the stabilization of the DNA-protein complex

(Kobayashi et al., 1995). This region shows some resemblance to those of the helix-loop-helix regulatory proteins and the leucine zipper transcription factors, which are known to interact directly with their cognate recognition sequences (Mitchell and Tjian, 1989; Johnson and McKnight, 1989; Fisher et al., 1991). There is also a possibility that the basic amino acid region has a role in the nuclear translocation of BTEB1, as previously demonstrated with the basic residues of Egr-1, another zinc-finger transcription factor (Gashler et al., 1993). BTEB1 contains two regions (region A, amino acid 13 to 26, region B, amino acid 58 to 68) responsible for its transcriptional activation function(s). These regions exhibit sequence similarities to each other but appear to enhance transcription independently (Kobayashi et al., 1995). Unlike other activation domains, such as the acidic (Ma and Ptashne, 1987), proline-rich (Mermod et al., 1989), and glutamine-rich (Courey and Tjian, 1988) domains identified for other transcription factors, the BTEB1 regions A and B are characterized by a high content of hydrophobic amino acids (Kobayashi et al., 1995). Numerous transcription factors such as VP16, NTF-1, and EBNA-2 (Regier et al., 1993; Cress and Triezenberg, 1991; Attardi and Tjian, 1993; Ling et al., 1993) have demonstrated the presence of hydrophobic amino acids which are important to transcriptional activation. BTEB1 acts as a transactivator of genes whose promoter regions contain repeated GC boxes, but as a trans-repressor in those whose promoter regions contains a single GC box; this is exemplified in the CYP1A1 gene promoter which is activated by Sp1 but repressed by BTEB1 (Imataka et al., 1992). This phenomenon further suggests that the transcriptional activity of BTEB1 on target genes involves the participation of other transcription factors, which may

function as coactivators or corepressors, and that the interactions of these factors with Sp1 can be different from those with BTEB1.

Expression of BTEB1. BTEB1 mRNA was found in all rat organs tested, similar to that of Sp1. However, the relative abundance of BTEB1 and Sp1 mRNAs varies among various organs. BTEB1 mRNA abundance is high in rat liver, lung, kidney, and especially in brain, whereas thymus and spleen contain a greater abundance of Sp1 mRNAs (Imataka et al., 1992). Interestingly, Sp1, unlike BTEB1, appears to be transcriptionally regulated since its protein concentration changes in parallel with that of its mRNA (saffer et al., 1990; Imataka et al., 1994). In a 1994 study by Imataka and colleages (Imataka et al., 1994), the presence of the BTEB1 protein was only shown in rat brain but not in testis, kidney, and lung although its mRNA was detected in all these organs. Furthermore, only a mouse neuroblastoma cell line, Neuro2A, and spermatocytes in the testis contained detectable BTEB1 protein; other cell lines, such as HeLa, CV-1, Cos-1, and NIH3T3, had undetectable immnoreactive BTEB1 in spite of the substantial abundance of BTEB1 mRNA in these cells (Imataka et al., 1994). In the female reproductive tract, the ovary, uterine endometrium, and placenta express BTEB1 mRNA, but the placenta has undetectable BTEB1 protein (Imataka et al., 1994). BTEB1 protein was localized preferentially to uterine endometrial epithelial cells (glandular and luminal epithelial cells) rather than stromal cells in the pregnant pig, although BTEB1 mRNA is detected in all three cell types (Wang et al., 1997). Taken together, these findings indicate that the expression of BTEB1 can be translationally regulated in a cell- or tissue-specific manner. Both rat and human BTEB1 mRNAs contain a long 5'UTR. The 5'-UTR of the human BTEB1 mRNA contains 10 upstream AUGs and a highly GC-rich content. The

latter, together with the first 95 bases of the BTEB1 coding region, is predicted to form a stable stem-loop structure (Yasumoto, 1993), which could lead to secondary structure and impede the 40S preinitiation complex from moving along the mRNA, thus repressing translation. In addition to translational regulation, BTEB1 expression can also be modulated at the level of transcription as demonstrated by the up-regulation of its mRNA levels by thyroid hormones acting through the thyroid hormone receptor β1 in neuro-2a cells (Denver et al., 1999).

Other KLF Members: BTEB2 and BTEB3

Members of the Krüppel-like family (KLF) of transcription factors are classified based on the three copies of two cysteine and two histidine zinc finger structural motif (C_2H_2) that they use to bind to DNA. These motifs exhibit remarkably conserved primary sequences. In addition, the KLF members share a highly conserved seven-amino acid inter-finger spacer, TGEKP(Y/F)X, often referred to as a H/C link (reviewed by Dang et al., 2000). At least 20 proteins are classified to belong to this family, and include Sp1-5, BTEB1-3 and other zinc-finger proteins. They play key roles in the regulation of a diverse range of biological processes, including cell growth, differentiation, embryogenesis, tumorigenesis, and apoptosis (reviewed by Black et al., 2001).

IKLF/BTEB2. BTEB2 was first isolated from a human placental cDNA library using rat BTEB1 cDNA as a hybridization probe (Sogawa et al., 1993). The open reading frame of BTEB2 encodes 219 amino acids, which represents only a part of the intestinal-enriched Krüppel-like factor (IKLF, KLF5, 446 amino acids), another KLF member (Conkright et al., 1999; Ohnishi et al., 2000). Like other family members, IKLF/BTEB2 has three C_2H_2 zinc finger motifs that can bind to CACCC sequences and a conserved

short sequence rich in basic amino acids immediately N-terminal to the zinc fingers. The N-terminal region of IKLF/BTEB2 is characteristically rich in proline residues (16% compare to 6% in Sp1 and BTEB1), and is implicated in protein-protein interactions (Williamson, 1994). Although BTEB2 encodes only a portion of IKLF (identity is 92%), their expression patterns are different. IKLF is expressed predominantly in the gastrointestinal tract, whereas BTEB-2 expression is less restrictive with expression seen in heart, placenta, lung, skeletal muscle, pancreas, bladder, prostate, in addition to the GI tract (Conkright et al., 1999). BTEB2 mRNA is rapidly and persistently induced by treatment with phorbol ester and basic fibroblast growth factor (Kawai-Kowase et al., 1999), suggesting a function for BTEB2 in mediating cellular responses to mitogenic stimulation. BTEB2 activates transcription of genes that contain multiple or single GC-box binding site(s) in their promoter regions, although activation is weak with promoters containing a single GC-box (Sogawa et al., 1993b).

RFLAT-1/BTEB3. BTEB3 is another transcription factor that belongs to the KLF family and is highly related to BTEB1. The mouse BTEB3 mRNA is ~5kb in length, and was initially detected in the developing as well as in the adult mouse, and in all tissues examined so far (Martin et al., 2000). *In vitro* synthesis of BTEB3 mRNA yielded a protein of 40 kDa consisting of 289 amino acids. Sequence analysis revealed that human and mouse BTEB3 proteins are identical in their primary sequences to the recently published sequences for human RFLAT-1 and FKLF-2 (Song et al., 1999; Kaczynski et al., 2001) and to mouse BTEB3 and KLF13 (Martin et al, 2000; Scohy et al., 2000), respectively. BTEB3 can bind to BTEs as well as to non-consensus GC box motifs. The mouse BTEB3 can activate transcription from the minimal promoter regions of genes that

are ubiquitously expressed (e.g., SV40) or exhibit tissue-restricted expression (e.g. SM22α) (Martin et al., 2000). However, a recent report by Kaczynski et al. (2001) indicated that the N-terminus of BTEB3 protein strongly repressed transcription in a dose-dependent manner when tethered to DNA through the heterologous DNA-binding domain of GAL4. Furthermore, they demonstrated that this repression by BTEB3 is due to its interaction with the co-repressor mSin3A and the histone deacetylase protein HDAC-1, as well as to its competition with Sp1 for BTE binding.

Steroid Hormone Receptors: Progesterone Receptors (PRs) and Estrogen Receptors (ERs)

PRs

Introduction. Progesterone (P) plays a pivotal role in female reproduction in mammals and during all stages of the ovarian cycle and pregnancy. It is involved in the control of ovulation, prepares the endometrium for implantation, regulates the entire implantation process, is responsible for the maintenance of pregnancy, and even regulates sexual behavior. The majority of progesterone effects occur as a result of progesterone-receptor (PR)-mediated action, where PR behaves as a hormone-dependent transcription factor. PR is a member of a large family of structurally-related gene products known as the nuclear receptor surperfamily.

Progesterone was discovered in 1934. Subsequent to its discovery, the time line for important events related to its function and mechanism of action include the following: 1) 1970: Baulieu and O'Malley's group reported that progesterone's action was likely mediated through a specific progesterone receptor protein (Milgrom and Baulieu, 1970; Sherman et al., 1970); 2) 1987: the human progesterone receptor was cloned by Misrahi et al. (Misrahi et al., 1987); and 3) 1990: the discovery of two human

PR isoforms, A and B, by Chambon's group expanded the functional significance of PR in physiological processes studies so far (Kastner et al., 1990). PR isoforms A and B are expressed from a single gene in rodents and humans as a result of transcription from two alternative promoters or of translation initiation at two different AUG codons. A functional promoter (located between –771 and +31) directs initiation of the human PR (hPR) B form mRNA from the authentic start sites located at +1 and +15. The second promoter initiating the hPR-A form is located between +464 and +1105. These two promoters utilize translation initiation AUGs that are located 165 codons away. Therefore, hPR-B, which contains 933 amino acids, exhibits an additional N-terminal fragment of 164 amino acids (Kastner et al., 1990). Six PR mRNA species with estimated sizes of 11.4, 6.1, 5.2,4.5,3.7, and 2.9 kb were detected in T47D cells. The 5.2 and 2.9 kb hPR RNAs are predominantly expressed, and correspond respectively to the B and A transcripts (Kastner et al., 1990). The protein sizes for hPR-B and hPR-A are 114 kDa and 94 kDa, respectively (Horwitz and Alexander, 1983; Carson-Jurica et al., 1990).

PR regulation The expression of PRs, and therefore target cell sensitivity to P, is under the control of estrogen, which increases, and progesterone, which decreases, PR mRNA levels in most P-responsive tissues (Graham and Clarke et al., 1997). During the follicular phase of the estrous cycle, high levels of PRs are present in the nuclei of epithelial and stromal cells of the human endometrium as well as in myometrial smooth muscle cells. In the mid- to late-luteal phase, levels of PRs in the luminal and glandular epithelium decline markedly. However, stromal and myometrial cells continue to express high levels of PRs despite high circulating P and the absence of estrogen receptors. Therefore, P does not always exhibit down-regulation of its receptor. The observed

decrease in PR levels in the breast between the follicular and luteal phases of the estrous cycle remains controversial (Joyeux et al., 1990). During pregnancy, PR expression declines equally in the uterine endometrium of several species, such as the bovine (Kimmins and MacLaren, 2001), equine (McDowell et al., 1999), rat (Ogle et al., 1998), and rabbit (Gutierrez-Sagal et al., 1993), and in the epithelial cells of the human endometrium (Perrot-Applanat et al., 1994). By contrast, mouse PR expression increases during early pregnancy, although this is restricted in the stroma at the sites of implantation (Tan et al., 1999).

Molecular structure and functional properties of PRs. The amino-terminal region of the PR (A and B) protein contains transactivation function domains (termed activation function, AF) that regulate the level and promoter specificity of target gene activation (Tora et al., 1988; Dobson et al., 1989; Meyer et al., 1992; Sartorius et al., 1994). AF-1 is localized in a 90-amino acid segment just upstream of the DNA-binding domain (DBD) and is found in both PR isoforms. AF-3 is localized within the unique 164 amino acid B-upstream segment and requires the functional DBD of hPR for its activity. Depending on the promoter or cell tested, AF-3 can activate transcription autonomously, or it can functionally synergize with AF-1 or AF-2, the latter is located within the C-terminal region of the PR protein (Sartorius et al., 1994). The highly conserved DNA binding domain, composed of two zinc fingers (see review by Carson-Jurica et al., 1990), is located centrally and contains sequences that contribute to receptor dimerization as well as specificity of DNA binding. Both PR and glucocorticoid receptor bound directly and specifically to the GRE/PRE, a symmetrical sequence (TGTACA and TGTTCT) separated by more than one base pair space (Strahle et al., 1987; Luisi et al., 1991;

Freedman, 1993). Downstream of the DNA binding domain lies a short hypervariable region containing the nuclear localization signals as well as an additional transactivation domain (AF-2) (Picard and Yamamoto, 1987; Hollenberg and Evans, 1988; Guiochon-Mantel et al., 1989; Dobson et al., 1989). The nuclear localization signal of PR is homologous to that of the SV40 large T antigen, and is located within amino acids 638-642. When this sequence segment was deleted, the receptor localized predominantly in the cytoplasm, however, the addition of the ligand shifted the cytoplasmic protein into the nucleus suggesting that the ligand-binding domain (LBD) also contributes to the nuclear localization of the receptors (Guiochon-Mantel et al., 1989). The LBD is located at the C-terminus of the PR protein. In addition to its progesterone binding function, it contains sequences important for the interaction of inactive receptors with cellular heat shock proteins, as well as sequences required for receptor dimerization, transactivation, and inter-intra molecular silencing (Pratt et al., 1988; Webster et al., 1988; Vegeto et al., 1992).

Mechanism of PR function. The PR has been shown to predominantly reside in the nucleus of target cells in a latent form in the absence of ligand (McDonnell, 1995). Under this condition, the receptor is associated with a high-molecular-weight complex composed of several heat shock proteins (hsp), which include hsp90, hsp70, and hsp56 (Pratt et al., 1988; Picard et al., 1988). However, PR can bind to DNA and transactivate target genes in a hormone-dependent manner (Bagchi et al., 1988; Bagchi et al., 1990). These results raise questions about the effects of heat-shock protein on the hormone dependency of PR function, and the roles of P beyond removal of heat-shock proteins. The PR-heat-shock protein complex is not a stable condition. Rather, it is in a dynamic

state in that the receptor is continuously diffusing out of, and being reimported into, the nucleus (Perrot-Applanat et al., 1992; Guiochon-Mantel et al., 1991). When P binds to the ligand-binding domain of PR, a PR/P complex structure composed of 12 α-helixes and β-turn motifs, a rapid transformation of the inactive receptor to an active state occurs. The interaction between P and the PR initiates a signal transduction cascade by promoting displacement of heat shock proteins and facilitating the formation of stable receptor dimers (McDonnell, 1995). Once dimerizarion occurs, the receptor is able to bind to DNA through its DNA-binding domains composed of two zinc fingers (Carson-Jurica et al., 1990). The fingers contain nine Cys molecules, and are proposed to interact with a half turn DNA (Evans, 1988). Carson-Jurica et al. (1990) determined that the specificity of binding by hormone response elements was specified mostly by the first zinc finger, and proposed that the second zinc finger may be involved with dimerization or nonspecific DNA interaction. A great deal of phosphorylation occurs after the receptor binds to DNA (Weigel et al., 1992; Bagchi et al., 1992; Takimoto et al., 1992), after which the active receptor complex interacts with specific coactivator proteins and general transcription factors to form a productive transcription initiation complex on specific target gene promoters (Onate et al., 1995; Kamei et al., 1996).

Recently, numerous proteins have been identified to be recruited by the liganded PR and enhance receptor-dependent transactivation. As discussed above, PR/P was shown to mediate the transactivation of the cyclin-dependent kinase inhibitor p21 gene promoter by forming a functional multiprotein complex with Sp1 and the transcription activator CBP/p300 (Owen et al., 1998). Steroid receptor coactivator-1 (SRC-1) was also found to interact with the PR in a ligand-dependent manner (Onate et al., 1995; Liu et al.,

2001), and the interaction is predominantly through the AF-2 containing LBD and/or AF-1 domain located in the N-terminus of PR (Onate et al., 1998). CREB-binding protein (CBP) and the histone acetyltransferase (HAT) p/CAF can also bind SRC-1, and these interactions allow SRC-1 to act as a bridge between PR and the general transcription machinery. Other co-activaters, such as TIF2, SRC-2, SRC-3, GRIP1 AIB1, L7/SPA, and HMG-1/2, also were shown to interact with PR, and to significantly impact the transactivation activity of PR (Voegel et al., 1996; Anzick et al., 1997; Hong et al., 1997; Jackson et al., 1997; Boonyaratanakornkit et al., 1998; Suen et al., 1998;). A unique coactivator for PR was recently identified. This coactivator, designated steroid receptor RNA activator (SRA), is a RNA transcript, while other steroid receptor coactivators are proteins (Lanz et al., 1999). SRA has a quite restricted pattern of expression, and has been largely detected only in the brain coincident with the expression of specific steroid hormone receptors. The finding that SRA exists in a complex with SRC-1 has led to the proposal that SRA directs coactivator complexes to the AF-1 region of steroid receptors and may serve as a scaffold upon which a multiprotein, functional complex is formed at the promoter site (Lanz et al., 1999).

PR/P can mediate cell proliferation or differentiation through cross-talk with a number of signaling cascades, which are triggered by growth factors and cytokines. Epidermal growth factor (EGF), via Ras/Mitogen-activated protein kinase (MAPK) activation, can regulate cyclin E only in P-pretreated mammary gland cells, where P potentiates the effects of EGF by up-regulating EGF receptor, c-ErbB2 and c-ErbB3 receptors, and by enhancing EGF-stimulated tyrosine phosphorylation of signaling molecules known to associate with activated type I EGF receptors (Lange et al., 1998).

P/PR also mediate STAT family activity at several levels. PR interacts with Stat5, and then promotes the nuclear translocation of Stat5 (Richer et al., 1998). In breast cancer cells, Stat5 and JAK2 are phosphorylated and/or recruited to phosphotyrosine-containing proteins after a single dose of P (Lange et al., 1998; Richer et al., 1998). Additionally, Stat5a, Stat5b, and Stat3 proteins are up-regulated while Stat1 is down-regulated, by P treatment in a PR-dependent manner (Richer et al., 1998).

Since PR-B contains an extra N-terminal 164 amino acid segment relative to PR-A, these isoforms are functionally different. When expressed individually in cultured cells, PRA and PRB display different transactivation properties that are specific to cell type and target gene promoter (Kastner et al., 1990; Vegeto et al., 1993; McDonnell, 1994; Hovland et al., 1998; Tora et al., 1998). Specifically, PR-B has been shown to function as a strong activator in certain gene promoters and specific cell types in which PR-A is inactive. The first 140 amino acid region of the human PR-A was identified to function as an inhibitory domain (ID), and this is responsible for the dominant transrepression of this PR isoform (Giangrande et al., 1997). Through this ID, human PR-A has a higher affinity for the corepressor SMRT than human PRB. In addition, human PR-A, unlike human PR-B, is unable to efficiently recruit the transcriptional coactivators GRIP1 and SRC-1 upon agonist binding (Giangrande et al., 2000). These findings indicate that PR isoforms A and B likely regulate different physiological target genes in response to P and each protein may display different transactivation capabilities in different target tissues. Furthermore, when the A and B proteins are coexpressed in cultured cells, where agonist bound PR-A is inactive, the protein can act as a dominant repressor of PR-B (Vegeto et al., 1993; Kraus et al., 1995). This repressor capability,

which appears to be a selective property of the PR-A protein, extends not only to PRB but also to other steroid hormones as well. PR-A has been shown to inhibit estrogen, glucocorticoid and mineralocorticoid receptor-dependent gene activation presumably through competition for common limiting coactivators, or by interfering with their ability to interact productively with the transcriptional machinery, a process known as quenching (McDonnell et al., 1994; Kraus et al., 1995).

Recent studies have demonstrated rapid non-genomic actions of P. Ca^{2+} influx, which is necessary for the acrosome reaction in human sperm, is rapidly stimulated by P (Thomas and Meizel 1989; Blackmore et al., 1990; Blackmore et al., 1991; Blackmore 1993). This action of P is mediated by a cell membrane receptor located in the Sperm head (Blackmore et al., 1990; Blackmore et al., 1991). In addition, studies showed that P had inhibitory non-genomic effects on Na^{+} absorption in Xenopus kidney epithelial cells (Fronius et al., 2001), and nitric oxide synthase activity was increased by acute, non-genomic effects of P on rat aorta, resulting in inhibition of platelet aggregation (Selles et al., 2001).

Physiological roles of PRs. Definitive proof that PRs are essential mediators of ovulation has been provided by the PR knock-out (PRKO) mouse (Conneely and Lydon, 2000). Conneely's group have established that PR is required specifically for LH-dependent follicular rupture leading to ovulation, but not for differentiation of granulosa cells to form the corpus luteum. Previous studies have indicated that both the PR-A and -B proteins are induced in preovulatory follicles in response to LH stimulation (Natraj and Richards, 1993). Stimulation of immature PRAKO mice, in which PR-A isoform is selectively ablated, with gonadotrophins indicated that superovulation is severely

impaired, but unlike PRKO mice is not completely absent. The observation that PR-B alone cannot support normal ovalation suggests that either the A protein is primarily responsible for follicular rupture or that this event is mediated by heterodimeric interactions between the A and B proteins (Conneely et al., 2001).

Female infertility in PRKO mice is also associated with defective uterine implantation and a lack of decidualization of uterine stromal cells in response to P (Lydon et al., 1995). Studies with the PRAKO mice indicated that expression of the PR-A protein in the uterus is required to mediate the decidulization response to P (Mulac-Jericevic et al., 2000). Moreover, several genes, namely calcitonin, histidine decarboxylase, amphiregulin, and lactoferrin, whose expression in the uterine epithelium are responsive to P and are associated with uterine receptivity, have been shown to be regulated distinctively by either PR-A or PR-B (Das et al., 1995; Paria et al., 1998; Zhu et al., 1998; McMaster et al., 1992).

Estrogen is the primary proliferative stimulus for uterine epithelium and its effects are inhibited by P (Clarke and Sutherland, 1990). Ablation of both the PR-A and PR-B isoforms in PRKO mice results in marked hyperplasia of the lumenal and glandular epithelial tissue due to the unopposed action of estrogen. The PRAKO mouse model revealed an unexpected ability of the PR-B protein to induce, rather than inhibit epithelial cell proliferation. The expression of PR-B protein alone in the uterus results in a gain of proliferation activity (Mulac-Jericevic et al., 2000). Therefore, uterine expression of the PRA isoform is required not only to oppose proliferation induced by estrogen but also that induced by P acting through the PR-B protein. This inhibitory activity of PR-A

observed in the uterus is tissue-specific and is not observed in the case of the mammary glands (Conneely et al., 2001).

During pregnancy, increased branching, alveolar proliferation, and differentiation of alveolar lobules in mammary gland is controlled primarily by P. The recent analysis of the mammary gland phenotype of PRAKO mice has demonstrated that PRB isoform is sufficient to elicit normal proliferative and differentiative responses of the gland to P in the absence of PRA (Conneely et al., 2000).

PRs also have the responsibility for coordinating the sexual behavior associated with reproduction to optimize the fertilization rates. In ovariectomized rats, P is required under physiological estrogen priming to induce lordosis (McEwen et al., 1987; Lauber et al., 1991). Preliminary analysis of the lordosis response in PRAKO mice supports an absolute requirement for PRA, and not for PR-B in the P-dependent lordosis response (Conneely et al., 2000).

ERs

Introduction. The ER-estrogen signaling system plays crucial roles in the developmental functions of the female reproductive tract, in the establishment of secondary sex characteristics and in reproductive behavior (Reviewed by Muramatsu and Inoue, 2000). Targeted disruption of ER gene indicated a critical role for ER in the normal development and reproductive physiology in the male population as well (Eddy et al., 1996). The naturally occurring estrogens (E) 17β-estradiol (E_2), estrone (E_1), and estriol (E_3) are C_{18} steroids derived from cholesterol. As regards the endogenous source of estrogens, the "two-cell" theory of estrogen synthesis is well-accepted. In this model, the ovarian theca cells secrete androgens that diffuse to the granulosa cells to be

aromatized to estrogens (Hillier et al., 1994). Estrone and estriol are primarily formed in the liver from estradiol. Like progesterone, the specific nuclear actions of estrogens are determined by the type of the hormone, the subtype or isoform of the estrogen receptor involved, the characteristics of the target gene promoter, and the balance of coactivators and corepressors that modulate the final transcriptional response to estrogen receptor-ligand complex (Reviewed by Gruber et al., 2002).

Molecular structure and functional properties of ERs. The ER, like other steroid hormone receptors, is a modular protein, with distinct, largely autonomous domains having specific functions such as ligand binding, dimerization, DNA binding, and transactivation. The centrally located DNA-binding domain (domain C) contains two zinc fingers that are involved in receptor binding (Schwabe et al., 1993) and dimerization (Jackson et al., 1993). The ER contains two distinct activation functions that can interact directly with factors of the transcriptional machinery: an N-terminal A/B domain containing activation function-1 (AF-1), and a hormone-dependent activation function-2 (AF-2), located in the ligand binding domain (domain E) along with the hormone binding function of ER. AF-1 and AF-2 work in a synergistic manner and are required for full ER activity in most cell contexts (Berry et al., 1990; Gronemeyer 1991; Kraus et al., 1995; Tzukerman et al., 1994; Kobayashi et al., 2000). The ligand-binding domain (domain E) contains different sets of amino acids that bind to different ligands (Mueller-Fahrnow and Egner, 1999). This domain also interacts with coregulatory proteins (Horwitz et al., 1996). The C-terminal domain contributes to the transactivation capacity of the receptor in a ligand-dependent manner.

Mechanism of ER function. The ERs, when unliganded, are loosely bound in its cytoplasmic or nuclear location to receptor-associated proteins. These proteins, which include hsp90 and hsp56 that also bind unliganded PR, serve as chaperones that stabilize the receptor in an unactivated state and/or mask the DNA-binding domain of the receptor (Smith and Toft, 1993). As free estrogen diffuses into the cell, it binds to the ligand-binding domain of the receptor, which dissociates from its cytoplasmic chaperones. These estrogen-ER complexes bind to estrogen-response elements (ERE) (5' GGTCAnnnTGACC 3', where n denotes a random nucleotide) (Walker et al., 1984) as homodimers or heterodimers between ER-α and ER-β, which will be discussed in the following section (Pettersson et al., 1997), and subsequently interact with the general transcription machinery. It has been shown that ligand-bound ER induces qualitative changes in the structure of the TBP/TFIID-TFIIB-DNA complex, resulting in the enhancement of subsequent basal transcription factor interactions (Sabbah et al., 1998). Similar to PRs, a number of coactivators, corepressors and other transcription factors interact with the ER molecule to modulate its transcriptional capacity. TIF1α, CBP/p300, SRC-1, CIP, NF-κB, Sp1 have all been shown to affect hormone-regulated promoter activity with ER (Ray et al., 1997; McKenna et al., 1999; Qin et al., 1999; Xie et al., 1999). Furthermore, estrogens also regulate the transcription of genes that lack functional ERE by modulating the activity of other transcription factors, as in the case with AP1 (Webb et al., 1995).

ER can also be activated in an E-independent manner. The activators of protein kinases including receptor tyrosine kinase (RTK) and MAPK, can activate ER by inducing its phosphorylation in the absence of ligand (Weigel 1996). Studies have shown

that these activators, which are involved in the cross-talk between distinct signaling pathways and ER, include dopamine, EGF, transforming growth factor (TGF) α, insulin, insulin-like growth factor (IGF)-I, heregulin, and cyclic AMP (cAMP) (Aronica and Katzenellenbogen, 1991; Smith et al., 1993; Ignar-Trowbridge et al., 1993; Newton et al., 1994; Pietras et al., 1995).

ER isoforms. ERα was first cloned in 1986 (Green et al., 1986), and is usually considered the dominant ER in most tissues. It is a 66 kDa protein consisting of 589 amino acids, and is expressed in major female organs such as ovary, uterus, vagina, mammary gland and certain areas of the central nervous system, especially in the hypothalamus (Muramatsu and Inoue, 2000).

The second estrogen receptor, ERβ, was cloned from rat prostate (Kuiper et al., 1996) and human testis (Mosselman et al., 1996). The human ERβ contains 530 amino acids (Muramatsu and Inoue, 2000). The genes encoding the two ERs are on different chromosomes. The ERα gene has been mapped to the long arm of chromosome 6, whereas the ERβ gene is located on chromosome 14 q22-24 (Gustafsson 1999). Although the DNA-binding domains of ERα and ERβ are very similar, the overall degree of homology of the receptors is low. This is particularly true for the ligand-binding domain, of which only 55% of the amino acid sequence is shared (Witkowska et al., 1997). Therefore, different ligands show different affinity to each of these receptor isoforms (Kuiper et al., 1997; Kuiper et al., 1998). Granulosa cells and developing spermatids contain mostly ERβ, and this subtype is present in several nonclassic estrogen target tissues, including the kidney, intestinal mucosa, lung parenchyma, bone marrow, bone, brain, endothelial cells, and prostate gland (Enmark et al., 1997). In a recent study, ERβ

was found in peri-implantation porcine embryos which lack detectable ERα expression; This ER isoform is postulated to mediate the autocrine functions of estrogens in the dynamic regulation of embryonic growth and development at implantation (Kowalski et al., 2002).

ERγ or putative estrogen receptor (pER) has been isolated and purified from mouse liver in 1998 (Rao 1998). ERγ is a serine phosphatase, but has high binding specificity for E_2, estrone and estriol. It was detected in the ovary, non-reproductive organs (skeletal, neural, vascular, hair and retina cells) and endometrial, prostatic and mammary tumours. ERγ may be involved in estrogen signaling pathway, and may contribute to numerous estrogenic actions in non-reproductive organs as well as participate in carcinogenesis in estrogen responsive tissues (Rao 1998).

A plasma membrane ERα has been identified on a GH3/B6 rat pituitary tumour cell line and several sublines that produce rapid (within minutes), non-genomic responses to E (Norfleet et al., 1999; Watson et al., 1999). Examples of effects mediated by this non-genomic pathway are the short-term vasodilation of the coronary artery (Kim et al., 1999), the rapid insulinotropic effect of E on pancreatic beta cells (Nadal et al., 1998), and the rapid activation of growth–factor-related signaling pathways (MAPK signaling cascade) in neuronal cells (Watters et al., 1997).

Physiological role of ERs. Estrogen is the primary proliferative stimulus for uterine epithelium (Clarke and Sutherland, 1990). It stimulates growth, blood flow, and water retention in sexual organs and is also involved in the etiology of breast cancer and endometrial cancer. Both male and female ERα-knockout mice are infertile (Lubahn et al., 1993). The female mice in which the gene for ERβ is knocked out are fertile and

exhibit normal sexual behavior, but have fewer and smaller litters than wild-type mice. Superovulation experiments indicate that this reduction in fertility is the result of reduced ovarian efficiency. Older male mice null for this gene display signs of prostate and bladder prostatic hyperplasis and loss of abdominal fat but are nevertheless fertile (Krege et al., 1998). In mice in which the genes for both ERα and ERβ are knocked out, the females have ovaries that contain seminiferous tubule-like structures that are filled with Sertoli-like cells, and the males are infertile because of a reduction in the number and motility of epididymal sperm – a phenotype similar to that in male ERα-knockout mice (Couse and Korach, 1999). In developing porcine embryos a surge in estrogen secretion is observed within a window of peri-implantation. The significance of this phenomenon maybe related to a novel role of estrogens in serving as the signal for maternal recognition of pregnancy in this species (Perry et al, 1973; Perry et al, 1976; Bazer and Thatcher, 1977; Gadsby et al, 1980; Heap et al, 1981).

Besides the documented functions of estrogen/ERs in the reproductive system, estrogens stimulate the growth and differentiation of the ductal epithelium in breast tissue, induce mitotic activity of breast ductal cylindric cells, and stimulate the growth of connective tissues (Porter 1974). In the central nervous system, the brain aromatization hypothesis proposes that sexual differentiation in the brain is dependent on the local conversion of androgens to estrogens; this process is mediated by two, separable brain aromatase systems in mammals, a gonad-sensitive hypothalamic system and a gonad-insensitive limbic system (Naftolin 1994). In later life, estrogens are thought to have neuro-protective actions (Garcia-Segura et al., 1999; Woolley et al., 1997; Green et al., 1997; Xu et al., 1998). Estrogens are also thought to be natural vasoprotective agents

since they can cause short-term vasodilation by increasing the formation and release of nitric oxide and prostacyclin in endothelial cells (Kim et al., 1999) and also reduce vascular smooth-muscle tone by opening specific calcium channels through a mechanism that is dependent on cyclic guanosine monophosphate (White et al., 1995). In addition, estrogens directly inhibit the function of osteoclasts, and estrogen deficiency is known to accelerate bone loss and increase susceptibility to fractures (Jilka 1998).

Implantation

Introduction

Implantation of the embryo into the endometrium is a critical step in the establishment of pregnancy, hence, the failure of embryo to implant is a major causative factor in the development of reproductive technologies. Implantation fundamentally involves four basic steps: (1) migration of the blastocyst into the uterine cavity; (2) attachment of the hatched blastocyst onto endometrial surface epithelium; (3) invasion of the trophoblast into subepithelial cell layers, a step which varies among species, and (4) growth of the trophoblast tissues which eventually form the placenta (Hill, 2001). These processes are fundamental to implantation in all species, and are analogous to patho-physiological processes involved in the progression of metastatic carcinoma, or in the endometrial implantation in ectopic locations as occurs as in endometriosis. Therefore, the elucidation of the important mechanisms behind the basic processes occurring during embryo implantation would be consequential to understanding tumor biology and gynecologic disorders.

The General Process of Implantation

The endometrium acquires the ability to allow implantation of the developing embryo within a specific time window, termed the "receptive phase". Just before implantation there is a distinctive change in the microvillar morphology of uterine luminal epithelial cell. Progesterone elicits shorter microvilli, and in several species, apical protrusions on luminal epithelial cell appear (Nilsson O, 1974; Dantzer, 1985; Martel et al., 1987; Martel et al., 1991). Psychoyos and co-workers have termed these protrusions "pinopodes" in rat and humans and suggest that these structures indicate a receptive uterine phase (Martel et al., 1987; Martel et al., 1991; Nikas et al., 1995). Attachment of the hatched blastocyst onto endometrial surface epithelium may consist of several steps involving initial low-affinity protein-carbohydrate-dependent systems (e.g., heparan sulfate), followed by higher-affinity, protein-protein interactions (e.g., integrins) (Armant et al., 1986; Lessey et al., 1992). If attachment occurs, the underlying stromal cells of many species are triggered to undergo a local differentiation reaction referred to as the decidual cell response (Bell, 1983; Parr and Parr, 1989). As a consequence, decidual cells undergo proliferation, hypertrophy and apoptosis as well as alter their patterns of expression of a variety of intra- and extracellular proteins. Following attachment, the final step, namely, invasion, must occur for successful implantation. This process is initiated by the enzymatic digestion of the extracellular matrix that simultaneously controls hemostasis and neoangiogenesis within decidual tissues (Chegini and Williams, 2000; Bischof et al., 2000). In this event, the key feature is the vascular development within the developing placenta and decidua, which allows the trophoblast to grow by the provision of an adequate blood supply.

The Role of Estrogen and Progesterone During Implantation

During implantation of the embryo, the uterine endometrium undergoes pronounced structural and functional changes. Estrogen initiates hypertrophy and hyperplasia of endometrial epithelia. Progesterone transforms this prepared endometrium into a secretory tissue and creates an environment within the uterine milieu that is conductive to embryo attachment (Yoshinaga, 1988; Weitlauf, 1994; Strauss and Gurpide, 1991). Steroid hormones act through their intracellular receptors, which are ligand-inducible gene regulatory factors (Evans, 1988; Beato, 1989; Tsai and O'Malley, 1994). It is therefore likely that steroids trigger the expression of a unique set of genes during the early stages of pregnancy and that these eventually lead to synthesis of new proteins that prepare the uterus to accept the invading blastocyst.

As mentioned above, estrogen contributes to uterine epithelial cell proliferation. Numerous growth factors, such as insulin-like growth factor-I (IGF-I), transforming growth factor-α (TGF-α) and epidermal growth factor (EGF), have been demonstrated to mediate estrogen-stimulated growth in the uterus (Sahlin, 1995; Hata et al., 1998; Zhang et al., 1998). The levels of IGF-I protein in the luminal fluid and uterine endometrium in the pig system were also increased by estrogen treatment (Simmen et al., 1990). Numerous evidences have been presented to indicate that estrogen induction of epithelial proliferation is a paracrine event mediated by ER-positive stromal cells (Cooke et al., 1997).

Progesterone regulates uterine stromal cell proliferation (Clarke and Sutherland, 1990). In addition, PRKO mice do not undergo the decidual cell reaction suggesting an essential role for P in uterine stromal cell differentiation (Lydon et al., 1995). Interestingly, this process is mediated by PR-A rather than PR-B (Mulac-Jericevic et al.,

2000). Colony stimulating factor-1, which was considered as absolutely necessary for implantation (Pollard et al., 1991), is up-regulated by progesterone in uterine stroma (Hatayama et al., 1994; Kariya et al., 1994). In uterine epithelium, the function of estrogen as the primary proliferative stimulus is inhibited by progesterone (Clarke and Sutherland, 1990). Therefore, progesterone is considered as a regulator of uterine epithelial cell differentiation. The expression of molecules, which are essential to implantation and are produced in uterine endometrial glandular epithelial cells, such as leukemia inhibitory factor (LIF) (Stewart et al., 1992; Chen DB et al., 1995; Liu et al., 2001) and calcitonin (Bagchi et al., 2001), are regulated by P. Additionally, in the porcine uterine endometrium, the synthesis of a number of epithelial cell products, which include secretory leukocyte protease inhibitor, and uteroferrin (UF), are under P-control (Fliss et al., 1991; Lamian et al., 1993; Reed et al., 1996; Simmen et al., 1999). However, recent studies have shown that the mechanism of regulation of pregnancy associated- gene expression by P is quite complicated and may be cell-context as well as protein-context dependent. The elucidation of the various mechanisms underlying P-control of individual gene targets will benefit not only the understanding of the distinct functions of E or P during pregnancy, but also the complex mechanisms behind tissue- and developmental-specific gene expression.

Species-specific Implantation

The process by which the trophoblast penetrates the uterine epithelium and makes contact with the maternal blood supply as well as the extent to which the trophoblast invades the maternal endometrium vary considerably among species. In rodents, which exhibit haemochorial placentation, apoptosis of the uterine epithelium occurs at the site

of blastocyst apposition prior to the trophoblast invasion of the stroma (Parr et al., 1987). In primates, including humans, which also exhibit haemochorial placentation, the cytoplasmic protrusions of the trophoblast interdigitate between the uterine epithelial cells and spread along the basement membrane underlying the luminal epithelium, before eroding this and invading into and through the stroma to eventually tap the blood vessels (Enders, 1997). In ruminants such as sheep and cattle, in which placentation is synepitheliochorial, the trophoblast does not invade through the uterine epithelium into the uterine stroma, although there is considerable tissue remodeling and development of new blood vessels. At implantation, fetal chorionic binuclear cells fuse with those of the uterine epithelium and the syncytial cells penetrate through the basal lamina (Wooding and Flint, 1994). In other species with epitheliochorial placentation, like the pig, there is also very little direct invasion of the trophoblast into the uterine stroma. Horses appear to be unique in that they have a subpopulation of highly invasive trophoblast cells forming the chorionic girdle, which penetrates through the uterine epithelium into the stroma to form endometrial cups (Enders and Liu, 1991).

Interestingly, trophoblast cells from most species appear to have a high degree of invasive capacity that can be demonstrated outside the uterine lumen or *in vitro*. Indeed, pig trophoblasts which are non-invasive in uterus are able to penetrate non-uterine epithelia and develop in ectopic sites (Samuel and Perry, 1972). The non-receptiveness of the uterine epithelium to a conceptus, with the exception occurring during the narrow window of a "receptive phase", as well as the presence of protease inhibitors likely underlie the restrained movement of the highly invasive embryonic tissue, with the level of restraint varying with the type of uterine environment.

Uterine Proteases and Their Inhibitors

Introduction

Proteases with a role in extracellular matrix (ECM) remodeling fall primarily into three categories: serine, cysteine and metallo-proteinases (Barrett, 1994). Since excessive or inappropriately timed proteolytic events need to be restrained, most known mammalian enzymes have partner protease inhibitors, which are often widely distributed in the same tissues in which the enzymes are likely to act.

Serine proteases are the largest class of mammalian proteases and mostly act at neutral pH. Among those produced in the uterus are urokinase-type plasminogen activator (uPA), plasmin (derived from plasminogen by the action of uPA), and kallikrein, which are produced mainly by endometrial fibroblasts (reviewed by Salamonsen, 1999). Many of these enzymes exert their action on matrix degradation, in part indirectly, by proteolytic activation of matrix metalloproteinases (MMPs). Specific inhibitors, such as plasminogen activator inhibitor-1 (PAI-1), PAI-2 and the protease nexin, which limit the action of PA, serve to balance the activity of many of these enzymes. Other serine protease inhibitors, such as secretory leukocyte protease inhibitor (SLPI) and uterine plasmin/trypsin inhibitor (UPTI) have been found in the porcine endometrium, and from cultured uterine endometrial glandular epithelial cells (Farmer et al., 1990; Cardenas et al., 1997). The gene expression of SLPI was regulated by estrogen and progesterone *in vitro* (Reed et al., 1996). Expression of uPA and PAIs is controlled mainly by transcriptional mechanisms, driven by hormones and growth factors in a cell type-specific manner (Vassalli et al., 1991).

Cysteine proteinases are lysosomal enzymes that act at acid pH. The best known are cathepsin B and cathepsin L, both of which cleave the N-terminal peptides of collagen that contain the covalent cross-links within and between molecules. Another member of this family, interleukin 1β-converting enzyme (ICE or caspase-1), releases active interleulin 1β (IL-1β) from its precursor, providing this cytokine for local actions including the regulation of transcription of MMPs (Tocci, 1997). The activity of cysteine proteinases is controlled by members of the cystatin superfamily that includes both intracellular (cystatin A and B) and extracellular (cystatin C) forms.

The MMPs, constitute the most critical enzymes for matrix degradation and remodeling, with activities that collectively allow the complete degradation of all components that comprise the interstitial matrix and basement membranes. The role of MMPs in tumor invasion and metastasis is well documented (Powell and Matrisian, 1996). The major groups of MMPs and their substrates include: collagenases (MMP-1), gelatinases (MMP-2, MMP-9), stromelysins and membrane-type MMPs (MT1-MMP). Most MMPs act extracellularly and at neutral pH and are released as latent precursors requiring activation by proteases such as plasmin, tryptase and elastase, as well as by other activated MMPs. Progesterone is a negative regulator of transcription of several MMPs including MMP-1, -3, and –9 (Salamonsen et al., 1997), although this can be overridden by some cytokines, including IL-1 and TNF-α (Singer et al., 1997; Zhang et al., 1998). Interestingly, the level of MMP-1, and -9 protein were decreased by the serine protease inhibitor, SLPI, through the latter's suppression of prostaglandin H-synthase-2 activity in monocytes (Zhang et al., 1997). In general, the family of tissue inhibitor of metalloproteinase (TIMPs) acts to restrain the action of MMPs by binding their active

forms in a 1:1 stoichiometry (Edwards et al., 1996). In addition to their protease inhibitor activities, TIMP-1 and TIMP-2 also exhibit growth factor activities (Hayakawa et al., 1992, 1994). Further embryotrophic (Satoh et al., 1994) and steroidogenic (Boujrad et al., 1995) actions have been described for TIMP-1, while TIMP-3 overexpression promotes apoptosis of vascular smooth muscle cells (Baker et al., 1998). These observations expand the functions and participation of protease inhibitors to biological processes not otherwise previously considered.

Secretory Leukocyte Protease Inhibitor (SLPI)

Introduction. Secretory leukocyte protease inhibitor (SLPI), also known as anti-leukoprotease inhibitor (ALP), human mucus proteinase inhibitor, and human seminal plasma inhibitor (HUSI-1), is a 12 kDa member of the chelonianin class of serine protease inhibitors which also includes SKALP/elafin (Francart et al., 1997). SLPI contains two homologous domains, with the amino-terminal domain representing the trypsin-binding and the carboxyl-terminal domain representing the elastase- and cathepsin G-binding regions, respectively. Each domain contains eight cysteines, which form disulfide bonds that comprise its characteristic four-disulfide core. The human SLPI gene is localized on chromosome 20q12-13. 2 and the mouse homologue on chromosome 2H, which are syntenic with each other (Kikuchi et al., 1998). Human SLPI mRNA is 0.7 kb, with a 399 bp open reading frame that encodes a 133 amino acid protein of Mr=12kDa. The combined nucleotide sequences of the porcine SLPI cDNA clones are approximately 600 nucleotides long, and encode a protein containing 114 amino acids. The deduced amino acid sequence of porcine SLPI is 68% similar in primary structure to that of human SLPI (Farmer et al., 1990). In most mammalian systems, SLPI is expressed

primarily in epithelial cells, especially secretory/glandular epithelial cells of numerous tissues including the human bronchi, skin, intestine, parotid and submandibular salivary gland, tonsillar and nasal mucosa, lacrimal gland, middle ear mucosa, breast, pancreas, male genital tract, uterine endometrium, first trimester decidua, trophoblast, cervix, fetal membrane, and amniotic fluid (De Water et al., 1986; Wiedow et al., 1998; Si-Tahar et al., 2000; Ohlsson et al., 1984; Westin et al., 1999; Maruyama et al., 1998; Carlsson et al., 1983; Semba et al., 1999; Nystrom et al., 1999; Ohlsson et al., 1995; King et al., 2000; Heinzel et al., 1986; Helmig et al., 1995), and mouse thymus, and spleen (Morita et al., 1999). In the pig, SLPI mRNA was detected in fetal and neonatal lung and small intestine, maternal lung, uterus, cervix, spleen, and small intestine (Farmer et al., 1990).

Functions of SLPI. SLPI exhibits diverse functions such as anti-inflammatory, anti-bacterial, anti-fungal, anti-viral, and even anti-infertility (Sallenave et al., 1997; Lentsch et al., 1999; Song et al., 1999; Gipson et al., 1999; Hiemstra et al., 1996; Tomee et al., 1998; Moriyama et al., 1998). Most of these functions have been correlated with SLPI's ability to inhibit proteolysis by virtue of its anti-elastase, -cathepsin G, -chymotrypsin, and -trypsin activities. Further, SLPI has been shown to regulate intracellular enzyme synthesis, suppress matrix metralloproteinase production and activity, mediate normal wound healing, prevent scar formation, and augment fertility (Zhang et al., 1997; Ashcroft et al., 2000; Sumi et al., 2000; Moriyama et al., 1998).

Roles in Reproduction In reproductive tissues, the synthesis of SLPI by the decidua, endometrium, trophoblast, amnion, and cervix, and its localization to adjacent cells and tissues are consistent with SLPI having a function correlated with specific pregnancy stages (Helmig et al., 1995; Denison et al., 1999; King et al., 2000). This function could

be largely related to SLPI's anti-inflammatory action on neutrophils and other inflammatory agents that infiltrate the uterine environment at pregnancy as well as to its anti-microbial activities. Furthermore, there is evidence to show that SLPI may play a crucial role in the control of implantation events during pregnancy. As previously described, there are two major kinds of placenta according to the extent of trophoblast invasion. Previous studies have shown that gene expression of SLPI is generally associated with species exhibiting the epitheliochorial type but not hemochorial type of placentation (Badinga et al, 1994). Moreover, a recent study has demonstrated that SLPI can inhibit the invasive potential of embryos *in vitro* (Xu et al., 2000).

The molecular mechanism underlying the diverse functions of SLPI in the reproductive system, especially during pregnancy, remains unclear. Numerous studies have suggested that SLPI may have additional functions other than the inhibition of protease activities. First, SLPI has been shown to suppress prostaglandin H-synthase-2 activity and consequently, levels of prostaglandin E_2 and cellular production of matrix metalloproteases in monocytes (Zhang et al., 1997). Second, similar to other protease inhibitors such as TIMPs that have growth-promoting activities (Hayakawa et al., 1992, 1994; Baker et al., 1998), SLPI also shows potential effects on cell proliferation. This protein supportes colony growth of human hematopoietic progenitor cells in the absence of serum (Goselink et al., 1996). SLPI also stimulates DNA synthesis in porcine endometrial glandular epithelial cells *in vitro* (Badinga et al., 1999). Further, SLPI has been shown to block nuclear factor kappa B (NF-Kappa B) activation (i.e. nuclear translocation) in rat lungs by increasing the levels of the NF-kappa B inhibitor, I-kappa-beta (Lentsch et al., 1999). NF-kappa B, which acts as a transcription factor, not only is a

crucial factor controlling the inflammatory response, but also regulates the cell cycle through cyclin D_1 (Guttridge et al., 1999; Joyce et al., 1999). The expression of SLPI was shown to be inversely correlated with the ability of macrophage cells to produce tumor necrosis factor α (TNF-α) in response to bacterial lipopolysaccharide (LPS) (Jin et al., 1998). Recently, studies with an SLPI knock-out mice indicated a negative linkage between SLPI and transforming growth factor-beta1 (TGF-β1), with SLPI KO mouse model exhibiting enhanced activation of this growth factor, compared to the wild-type during wound healing (Ashcroft et al., 2000). Related to these observations, both TNF-α and TGF-β1 are well-known factors that inhibit cell proliferation.

Summary

Implantation of the embryo into the receptive endometrium is a highly coordinated process that involves the differentiation of uterine endometrium, development of embryo, and the establishment of correct maternal-embryo interactions. The genes that are specifically expressed during this time are referred to as pregnancy-associated, and their encoded proteins, which include growth factors, proteases, proteases inhibitors, cytokines, regulate the implantation process. It has been shown previously that BTEB1, a member of the Sp1/Krüppel-like family of transcription factors, regulates the expression of numerous pregnancy-associated genes including UF and SLPI. However, the exact role of BTEB1 during implantation and pregnancy remains unclear, specifically within the context of the pregnancy hormones, E and P. Since uterine endometrial epithelial cells must undergo specific proliferation and differentiation events in preparation for embryo implantation, and since BTEB1 has been demonstrated, albeit in a limited sense, to regulate P-dependent gene expression as well as cell proliferation, it is

likely that BTEB1 plays a significant role in the molecular events associated with implantation and pregnancy success. However, what are the molecular mechanisms beyond these observations remains unknown. The present studies were undertaken to further elucidate the complex mechanisms underlying uterine endometrial epithelial cell proliferation and differentiation as mediated by BTEB1. These studies are described in the following chapters.

CHAPTER 3
STEROID HORMONE REGULATION OF Sp1 AND BTEB1 EXPRESSION IN UTERINE ENDOMETRIAL EPITHELIAL CELLS

Introduction

Implantation of the embryo in the endometrium is a critical step in the establishment of pregnancy. This is a complex and highly coordinated series of events marked by alterations in the development of the embryo, differentiation of the uterine endometrium, and establishment of essential embryo-maternal interactions. These events are primarily controlled by the ovarian steroids, progesterone (P) and estrogen (E), and are mediated by a myriad of signaling molecules, which include proteases, protease inhibitors, growth factors, and cytokines, each with specific spatial and temporal modes of expression. However, the differential pattern of synthesis of these signaling molecules and the effects of ovarian steroids on their synthesis are not well understood.

Gene expression is regulated largely at the level of transcription. Transcription factors have critical roles in this event, and exert biological responses at the gene level (Tsai and O'Malley, 1994; Blackwood and Kadonaga, 1998). Basic transcription element binding protein (BTEB1/KLF9) and Sp1 are members of the Sp/KLF family. This family of proteins binds GC-rich motifs widely distributed within gene promoters through their C_2H_2 zinc finger DNA-binding domains, resulting in distinct activation or repression of transcriptional activities. BTEB1 expression is transcriptionally and translationally regulated in a cell-specific or tissue-specific manner (Imataka et al, 1994). Functional

BTEB1 protein was detected in endometrium of early (Days 12 and 21), mid (Day 60), and late pregnant (Day 90) pigs (Wang et al, 1997). More interestingly, BTEB1 mRNAs were detected in glandular epithelial (GE), luminal epithelial (LE) and stromal (ST) cells isolated from Day12 pregnant pig endometrium, but only GE and LE cells contain the functional BTEB1 protein (Wang et al, 1997). Sp1 mRNA and functional protein were also detected in the GE and LE cells obtained at different stages of pig pregnancy (Simmen et al, 2000). Therefore, BTEB1 and Sp1 may actively participate in the regulation of pregnancy-associated genes.

Although Sp1 is usually considered as a "housekeeping" transcription factor, recent studies indicate that P regulates the Tissue Factor gene in human endometrial stroma cells through the induction of Sp1 and the concomitant inhibition of Sp3 gene expression (Krikun et al., 2000). Members of the KLF family, however, appear to be more sensitive to hormonal control, consistent with their presumed distinct tissue-, cell- and developmental- associated expression. A recent study has shown that BTEB1 gene expression was induced by thyroid hormone in Neuro-2A cells, in agreement with the observed increase in the levels of BTEB1 mRNA in developing rat brain (Denver et al., 1999).

To investigate a role for uterine BTEB1 and Sp1 during peri-implantation, the regulation of these respective genes expression by E and P was investigated using the pig uterine endometrium. Days (D) 10 to D17 of gestation in the pig represent the time when developing embryos initiate attachment to the maternal endometrium. At around D11 and D12, intra-luminal E reaches maximal levels, afterwhich time, a slight drop in levels is observed at D14, followed by a modest increase at D18 (Geisert et al, 1990). In parallel,

plasma P reaches its highest concentrations at D11 and D12, decreases to a constant level by D20, and is maintained at this level until just before parturition. Therefore, both E and P hormonal levels exhibit dramatic changes around D10 and D14, the period in which the uterus and embryo must cooperate to allow firm adhesion of trophoblast to uterine epithelium (Morgan et al, 1987; Keys and King, 1990). Pig uterine endometrial glandular epithelial (GE) cells at pregnancy D11 and D14, which represent high (D11) and low (D14) E and P concentrations, respectively (Geisert et al, 1990), were used in this study to investigate the regulation of BTEB1 and Sp1 gene expression by steroid hormones, E and P.

Materials and Methods

Reagents were obtained as follows: Taq DNA polymerase from Boehringer Mannheim (Indianapolis, IN); nick-translation kit from Amersham Pharmacia Biotech Inc. (Piscataway, NJ); complementary DNA (cDNA) cycle Kit from Invitrogen (San Diego, CA); [α-32P]deoxycytidine triphosphate(3000 Ci/mmol), Biotrans nylon membranes(0.2 μm) from ICN Radiochemicals (Irvine, CA); Total cellular RNA isolation reagent TriZol from GIBCO-BRL (Gaithersburg, MD), and cell culture media from GIBCO-BRL (Grand Island, NY). All molecular biology-grade chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA).

Animals, Tissue Collection and Primary Cell Culture

Pigs were bred at estrus (Day 0) after two estrous cycles of normal duration (18-22 days) were observed. Animals were slaughtered at the Meats Processing Facility (University of Florida), and reproductive tracts were immediately removed and placed in ice. Glandular epithelial (GE) cells were isolated from freshly collected endometrium, as

previously described (Reed et al., 1996). Some GE cells were upon isolation, put into TriZol and stored at -80°C until further analysis. Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 0.25 U/ml insulin, and 1% antibiotic-antimycotic drugs, at 37°C in a 95% (v/v) air:5% (v/v) CO_2 environment. When GE cells reached ~80% confluence, they were preconditioned for 24 hours in fresh RPMI 1640 medium containing 10% (w/v) charcoal-stripped FBS. Fetal bovine serum treated in this manner has undetectable levels (<1pg, as determined by RIA) of estradiol and progesterone (Reed et al., 1996). The cells were then incubated in fresh medium containing 10% (w/v) charcoal-stripped FBS with or without estradiol (E_2, 1, 10, or 100nM), progesterone (P, 1, 10, or 100nM), or E_2 (100nM)+P (100nM). After 24 hours, cells were harvested and processed for RNA isolation. GE cells that were subjected to nuclear extract preparation were also isolated from D10 pregnant pig uterine endometrium. After preconditioning for 24 h in 10% (w/v) charcoal-stripped FBS medium, cells were incubated with regular medium containing 10% FBS with or without (ethanol), E_2 (100nM), P (100nM) or E_2 +P (100nM+100nM). Cells were harvested after 1h or 24h, and frozen at –80°C for later nuclear protein extraction. The experiments on D11 and D10 cells utilized three pregnant pigs, and the experiments on D14 cells utilized five pregnant pigs (each pig = 1 replicate).

RNA Isolation

Total cellular RNA from uterine endometrial GE cells was isolated using TriZol, following the manufacturer's instructions. The yield and quality of the isolated RNAs were assessed by the 260/280 nm optical density ratio and by electrophoresis under denaturing conditions in a 2% agarose gel.

RT-PCR

cDNA was synthesized from total RNA (5μg) prepared from freshly isolated or steroid hormone treated GE cells using the cDNA cycle kit. PCR amplification for BTEB, Sp1, UF, PR and β2-Microglobulin (β2M) was performed in a total volume of 50μl containing 50 pmol of the forward and reverse primers (Wang et al.,1997; Simmen et al., 2000), 10nM of each dNTP, 1 X PCR Buffer, Taq polymerase, and cDNA template (Table 3-1). The number of cycles and the amount of cDNA template for each PCR reaction was optimized so that amplification of the products was in the exponential phase and the assay was linear relative to the amount of input RNA. All PCR products were separated in 1.2% agarose gels containing ethidium bromide, and were visualized over UV light.

Southern Blot Analysis

Since BTEB and Sp1, being transcription factors, are present in low abundance, their PCR products under the optimized conditions were still only marginally detected in 1.2% agarose gels containing ethidium bromide. We therefore used Southern blotting to amplify the PCR product signals. After electrophoresis, the gel was soaked in 0.2N HCl solution for 10 minutes, followed by Denaturation Solution (1.5M NaCl, 0.5M NaOH) and Neutralization Solution (3M NaCl, 0.5M Tris). DNA was then transfered from the pre-treated gel to a Nylon membrane overnight. After crosslinking over the UV light for 90 seconds, the membrane was prehybridized for 2 hours at 65°C in 6 x SSC, 0.5% SDS, and 1 x Denhardt's solution. Purified PCR products were labeled by nick translation (Nick translation kit, N 5000; Amersham Pharmacia Biotech Inc.) After pre-hybridization, the membrane was incubated in the Hybridization Buffer (6 x SSC,

Table 3-1. Primers and conditions used in PCR reactions

Location		Primer Sequence 5'-3' and Condition *	Size of product (bp)
UF	sense	GACCTGGCTCGCCTTGG	812
	anti-sense	GCGATTGCCACAACTGTG	
		95°C/2min; 55°C/2min; 72°C/3min; 30 cycles; Buffer F	
PR	sense	GGGAATTCGTTTCACCATCCCTGCCAATATCT	290
	anti-sense	GGCTCGAGTCATTACCTCAGAAGATTTGTT	
		95°C/2min; 55°C/2min; 72°C/3min; 30 cycles; Buffer F	
BTEB	sense	ACTTGTGCCTCTTTTCGG	416
	anti-sense	CCTACATGGACTTCGTGG	
		95°C/1min; 55°C/1min;72°C/1min; 30 cycles; Buffer F	
Sp1	sense	GCCTCCAGACCATTAAC	717
	anti-sense	GCATGACGTTGATGCCA	
		95°C/1min; 55°C/1min; 72°C/1min; 30 cycles; Buffer A	
β_2 M	sense	CTGCTCTCACTGTCTGG	287
	anti-sense	ATCGAGAGTCACGTGCT	
		95°C/1min; 55°C/1min;72°C/1min; 30 cycles; Buffer F	

* In all PCR reactions, template and primers were denatured at 95°C for 2 minutes before thermal cycling began, and an additional extension cycle was performed for 7 minutes at 72°C before cooling the reaction mixture to 4°C .

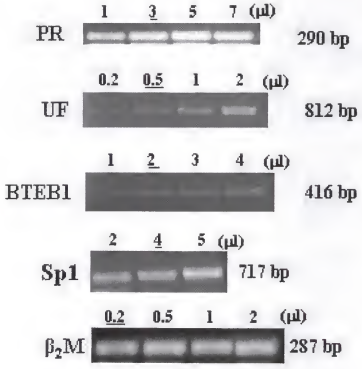


Figure 3-1. Optimization of PCR conditions. Total RNA was isolated from D11 pregnant pig uterine endometrial GE cells without steroid hormone treatment, and 5μg total RNA was subjected to reverse transcription. Under the conditions described in Table 3-1, the PCRs for PR, UF, BTEB1, Sp1 and b2M were conducted using different amounts of cDNA template (1:10 dilution). The PCR products were separated in 1% agarose gels containing ethidium bromide, and were visualized over UV light. The template amount selected for use in subsequent PCR is underlined.

0.5%SDS, 1 x Denhardt, 1mM EDTA) with purified probe at 65°C overnight. Following hybrization, the membrane was washed in 2 x SSC/0.1%SDS and 0.1 x SSC/0.1%SDS solution 2 x each, and exposed to X-ray film for 3 hours at –80°C.

Isolation of Nuclear Protein Extracts and Western Blot Analysis

Preparation of nuclear protein extracts followed the protocol from Dr. Peter Barnes's laboratory. The steroid hormone-treated GE cells were homogenized with a glass homogenizer in 5ml of buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.1% Nonidet P40). After centrifugation at 1500 rpm for 5 min, the nuclear pellet was lysed with 60μl of buffer B (20 mM HEPES, 1.5 mM $MgCl_2$, 0.42 mM NaCl, 0.5 mM DTT, 25% glycerol, 0.5 mM phenylmethylsulfonyl flouride, PMSF, and 0.2 mM EDTA), and the lysate was then centrifuged at 1500 rpm for 5 min. The soluble fraction was mixed with 100μl buffer C (20 mM HEPES, 50 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 0.2 mM EDTA), aliquoted in 20μl volumes, and frozen at –80°C immediately. Protein concentration of extracts was determined using the Bradford method (Bradford, 1976). Nuclear extract proteins (30μg/sample) were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with anti-BTEB1 (1:500 dilution, Zhang et al., 2002), or anti-Sp1 (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) antibody. The ECL system was used to visualize immunoreactive proteins (Amersham Pharmacia Biotech Corp., Arlington Heights, IL).

Statistical Analysis

All numerical data were obtained from densitometric analysis of PCR gels or Southern and Western blots. Each value representing the treatment sample was divided by that representing the control band, and was analyzed using ANOVA following the

general linear models procedures of the Statistical Analysis System. Comparisons between paired groups were analyzed with Students's *t*-test. Values were considered significant at $p<0.05$ and are presented as means ± SE.

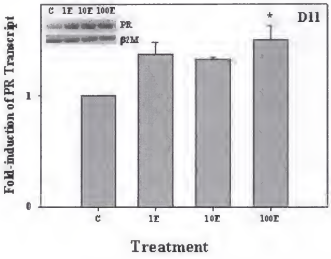
Results

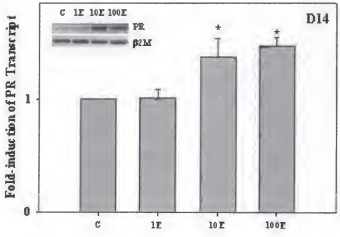
Progesterone Receptor (PR) mRNA Levels in Response to Different Concentrations of Estrogen in Glandular Epithelial Cells fron D11 and D14 Pregnancy Endometrium

To examine the expression of a number of genes at the mRNA level in steroid hormone treated GE cells, which did not yield enough total cellular RNA for extensive Northern Blot analyses, we used the more sensitive RT-PCR methodology to evaluate mRNA abundance. Each PCR reaction was optimized for a number of parameters (Table 3-1) and for template input (Figure 3-1), so that amplification was in the linear phase. To confirm that the cDNA concentration was consistent between different treatments in each experiment, we performed PCR for β_2M in parallel (control gene), and compared results for each gene with that for β2M within each cDNA preparation.

PR mRNA expression, which is well-known to be upregulated by estrogen, was used to verify the responsiveness of GE cells to E_2 treatments. D11 and D14 pregnant pig uterine endometrial GE cells were treated with E_2 (1, 10, 100nM) for 24 h after preconditioning of these cells in charcoal-stripped FBS-containing medium. The abundance of PR mRNA in these cells was examined using RT-PCR. After normalization to the β_2M value, each PR value was divided by the control value (no E_2 treatment) to yield the fold induction (mean ± SD). Figure 3-2 shows that PR mRNA expression increased after estrogen treatment at the highest dose. In D14 GE cells, both 10nM and 100nM E_2 treatments resulted in increased PR mRNA abundance ($p<0.05$) (Figure 3-2, lower panel); In

Figure 3-2. Effect of estrogen on PR mRNA abundance in GE cells. D11 (upper panel) and D14 (lower panel) pregnant pig uterine endometrial GE cells were treated with 1, 10, 100nM or no 17β-estradiol for 24 hours after preconditioning of these cells in charcoal-stripped FBS-containing medium. PR and β_2M mRNAs were monitored by RT-PCR. All numerical data were obtained from densitometric analysis of PCR products. After normalization to the β_2M value, each PR value was divided by the control value (no E2 treatment) to yield the fold induction (Lsmeans±SD). Each experiment was repeated on cells isolated from three (D11) or from five (D14) different animals. *, statistically significant ($P<0.05$) compared with control.


C 1E 10E 100E
PR
β2M
D11
*
Fold-induction of PR Transcript
1
0
C
1E
10E
100E
Treatment


C 1E 10E 100E
PR
β2M
D14
*
*
Fold-induction of PR Transcript
1
0
C
1E
10E
100E
Treatment

contrast, only higher concentration of E2 (100nM) increased PR mRNA in GE cells isolated from D11 pregnant pig endometrium ($p<0.05$) (Figure 3-2, upper panel). Representative PCR results are shown for each panel.

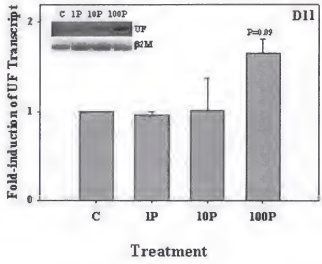
Uteroferrin (UF) mRNA Levels in Response to Different Concentrations of Progesterone in Glandular Epithelial Cells from D11 and D14 Pregnancy Endometrium

The gene encoding uteroferrin (UF), a porcine transplacental iron transport protein (Roberts and Bazer, 1980) that belongs to a family of tartrate-resistant acid phosphatases in mammals (Drexler HG and Gignac SM, 1994), is transactivated by P in porcine endometrium *in vivo* and *in vitro* (Malathy et al., 1990; Fliss et al., 1991; Simmen et al., 1991; Lamian et al., 1993; Simmen et al., 1999). In the present study, UF mRNA was used as an index to verify the P-responsiveness of GE cells prepared from D11 and D14 pregnant pig uterine endometrium. These cells were treated with P (1, 10, 100nM) for 24 h in 10% FBS containing medium. The UF mRNA abundance was measured by RT-PCR, and UF mRNA changes were analyzed following protocol described for PR mRNA. The results show that the abundance of UF mRNA tended to increase ($p=0.09$) with the 100nM P treatment in D11 pregnant pig GE cells (Figure 3-3, upper panel). However, there was no change in UF mRNA abundance after treatment of GE cells isolated from D14 pregnant pig endometrium with P (Figure 3-3, lower panel).

BTEB1 and Sp1 mRNA Levels in Response to Estrogen and Progesterone in Endometrial Glandular Epithelial Cells

GE cells that were isolated from D11 and D14 pregnant pig uterine endometrium were treated with different concentrations (0, 1, 10, 100nM) of E_2 and P or E_2+P (100nM E_2 + 100nM P) for 24 h after being preconditioned in charcoal-stripped FBS-containing

Figure 3-3. Effect of progesterone on UF mRNA abundance in GE cells. D11 (upper panel) and D14 (lower panel) pregnant pig uterine endometrial GE cells were treated with 1, 10, 100nM or no progesterone (P) for 24 hours. UF and β_2M mRNAs were monitored by RT-PCR. Numerical data were obtained from densitometric analysis of PCR bands. After normalization to the β_2M value, each UF value was divided by the control value (no P treatment) to yield the fold induction (mean±SD). Each experiment was repeated on cells isolated from three (D11) or five (D14) different animals.


C 1P 10P 100P
UF
β2M
D11
P=0.09
Fold-induction of UF Transcript
2
1
0
C
1P
10P
100P
Treatment

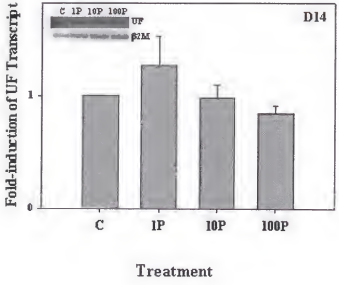
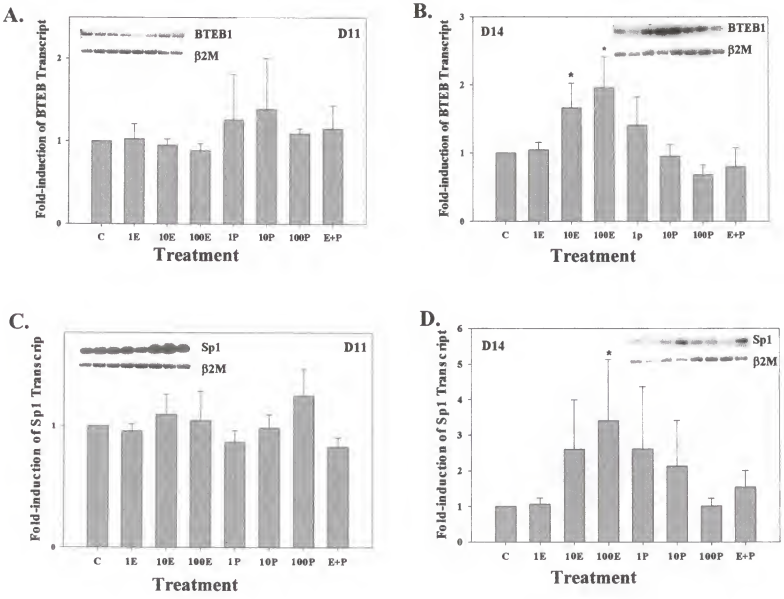

C 1P 10P 100P
UF
β2M
D14
Fold-induction of UF Transcript
1
0
C
1P
10P
100P
Treatment

Figure 3-4. Estrogen and progesterone effects on endometrial GE cell BTEB and Sp1 mRNA levels. D11 and D14 GE cells were treated with 17β-estradiol (E2) (1, 10, 100nM), progesterone (P)(100nM) or E2 ± P (100nM each) for 24 hours after preconditioning of these cells in charcoal-stripped FBS-containing medium. BTEB and Sp1 mRNAs signals were amplified by Southern blot using their RT-PCR product as probe. Numerical data were obtained from densitometric analysis of Southern blot bands. After normalization to β_2M PCR product in the EtBr-stained gels, BTEB or Sp1 values were divided by control values (no E2 or P treatment) to obtain the fold-induction (mean±SD). BTEB RT-PCR product (416bp) abundance in D11 (panel A) and D14 (panel B) GE cells after treatment with different concentrations of E2, P or E2 ± P are shown. Sp1 RT-PCR product (717bp) abundance in D11 (panel C) and D14 (panel D) GE cells after treatment with different concentrations of E2, P or E2 ± P are shown. Experiments for Day 11 were performed on cells isolated from three animals, and experiments for D14 were on cells isolated from five animals. *, statistically different ($P<0.05$) compared with the control.


A.
BTEB1
β2M
D11
Fold-induction of BTEB Transcript
C
1E
10E
100E
1P
10P
100P
E+P
Treatment
B.
D14
BTEB1
β2M
Fold-induction of BTEB Transcript
C
1E
10E
100E
1p
10P
100P
E+P
Treatment
C.
Sp1
β2M
D11
Fold-induction of Sp1 Transcrip
C
1E
10E
100E
1P
10P
100P
E+P
Treatment
D.
D14
Sp1
β2M
Fold-induction of Sp1 Transcript
C
1E
10E
100E
1P
10P
100P
E+P
Treatment

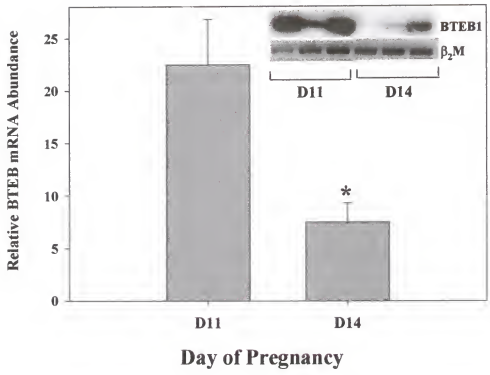
medium. These cells were subjected to total RNA isolation, and their BTEB1 and Sp1 mRNA abundance were compared by RT-PCR and subsequent Southern blot analysis. In D11 GE cells, BTEB1 and Sp1 mRNA abundance were unaffected by any of the treatments ($P>0.05$) (Figure 3-4 A, C). However, in GE cells isolated from D14 pregnant pig uterine endometrium, BTEB1 mRNA abundance was significantly increased by E_2 (10nM and 100nM) when compared to control (0 nM E_2), while Sp1 mRNA abundance was also increased but only by treatment with the higher E_2 concentration (Figure 3-4 B, D). However, P or E + P had no effect on the expression levels of either transcription factor in D11 and D14 pregnant pig GE cells (Figure 3-4).

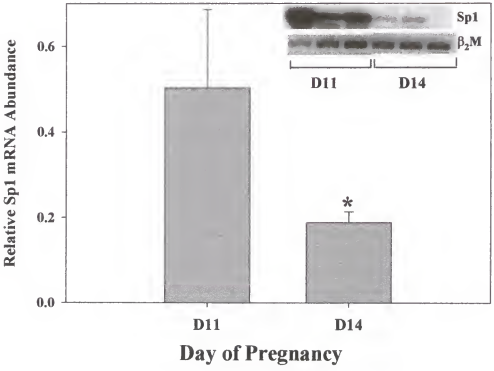
Basal BTEB1 and Sp1 mRNA Levels in Endometrial Glandular Epithelial Cells

Figure 3-4 (above) shows that BTEB1 and Sp1 gene expression in GE cells isolated from D11 or D14 pregnant pig uterine endometrium were differentially affected by E_2 treatment. As previously described, GE cells *in vivo* are in a higher E_2 environment at D11 than at D14 of pregnancy. We determined the basal levels of BTEB1 and Sp1 mRNA in freshly isolated GE cells from both pregnancy days to examine their *in vivo* expression levels. Total RNA was freshly isolated from D11 and D14 pregnant pig uterine endometrium GE cells, and subjected to BTEB1 and Sp1 RT-PCR and Southern blot analyses. Figure 3-5 shows that the mRNA abundance of both BTEB1 (upper panel) and Sp1 (lower panel) was significantly higher ($P<0.05$) in D11 than in D14 GE cells.

Estrogen and Progesterone Effects on BTEB1 and Sp1 Protein Levels in Endometrial Glandular Epithelial Cells

Figure 3-5. BTEB1 and Sp1 mRNA levels in uterine endometrial GE cells as a function of day of pregnancy. Total cellular RNA was extracted from freshly isolated GE cells from endometrium of individual animals at D11 and D14 of pregnancy (n=3 animals per day). Steady-state levels of BTEB1 (top panel) and Sp1 (bottom panel) mRNAs were compared by Southern blot using RT-PCR. The signals obtained from Southern blot were quantified by densitometric scanning and normalized to that of β_2M RT-PCR product obtained using the same cDNA preparations. The sizes of the expected products for BTEB1, Sp1 and β_2M are 416 bp, 717 bp, and 287 bp, respectively. *, statistically different ($p<0.05$) by Student's t test.


Relative BTEB mRNA Abundance
25
20
15
10
5
0
BTEB1
β2M
D11
D14
*
D11
D14
Day of Pregnancy

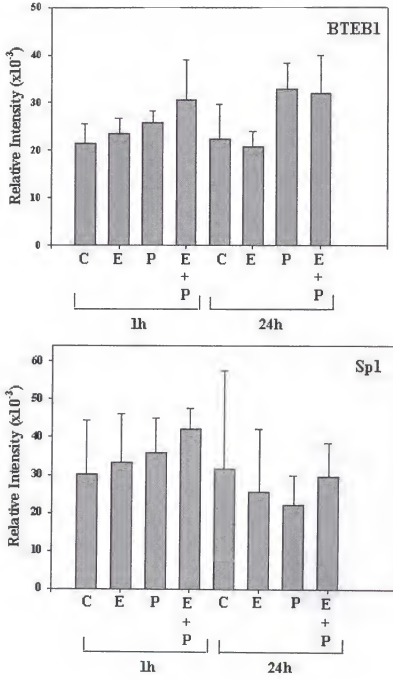

Relative Sp1 mRNA Abundance
0.6
0.4
0.2
0.0
Sp1
β2M
D11
D14
*
D11
D14
Day of Pregnancy

Since transcription factors function through protein-DNA or protein-protein interactions, levels of BTEB1 and Sp1 protein were also measured in the current study. Western Blot analysis was performed on nuclear extracts prepared from GE cells of D10 pregnancy endometrium previously treated with E_2 (100 nM), P (100 nM), or E_2 + P (100 nM + 100 nM) for 1h or 24h. Figure 3-6 shows that the abundance of both BTEB1 (upper panel) and Sp1 (lower panel) protein was unchanged with steroid hormone treatments at the time points.

Discussion

To address the roles of the two Sp/Krüppel family members, Sp1 and BTEB1, in uterine endometrial gene expression during peri-implantation, and to examine the relationship between these two transcription factors and the pregnancy hormones, E and P, the present study investigated the effects of E and P on Sp1 and BTEB1 gene expression using the pig uterine endometrial GE cell model. Results from the mRNA analyses showed that BTEB1 and Sp1 mRNA levels are increased by E treatment (10 and 100 nM E_2 for BTEB1, and 100 nM E_2 for Sp1) in GE cells isolated from D14 pregnant pig uterine endometrium (low *in vivo* E background), but not from D11 pregnant pig uterine endometrium (high *in vivo* E background). However, the levels of BTEB1 and Sp1 protein in GE cells isolated from D10 pregnant pig endometrium did not change after E treatment although the pig endometrium was never exposed to E at that pregnancy stage. These observations suggest that BTEB1 and Sp1 gene expression is under transcriptional as well as translational regulation. Previous studies have indicated that BTEB1 expression is translationally regulated in both rat and human tissues as a consequence of their BTEB1 mRNAs exhibiting long 5'UTRs which contain 10 uAUGs

Figure 3-6. Estrogen and progesterone effects on BTEB1 (upper panel) and Sp1 (lower panel) protein levels in D10 pregnant pig uterine endometrial GE cells. Primary culture of GE cells isolated from D10 pregnant pigs were treated with 17β-estradiol (E2) (100nM), progesterone (P) (100nM), E2 ± P (100nM each) or without steroid hormone (C) for 1 or 24 hours after preconditioning of these cells in charcoal-stripped FBS-containing medium. Nuclear extracts were isolated from these cells and 30μg nuclear protein from each sample was separated in SDS-PAGE. BTEB1 antibody (1:500 dilution) and Sp1 antibody (1:1000 dilution) were used for the Western blot analyses. Numerical data were obtained from densitometric analysis of immunoreactive bands. The experiments were repeated four times, and each treatment value (means±SD) was compared to the control value. Statistical differences were considered to be significant at $p<0.05$ by Student's t test.


BTEB1
Relative Intensity (x10^{-3})
50
40
30
20
10
0
C E P E + P C E P E + P
1h
24h
Sp1
Relative Intensity (x10^{-3})
60
50
40
30
20
10
0
C E P E + P C E P E + P
1h
24h

within a highly GC-rich region to form a stable stem loop structure (Imataka et al., 1994). The present study provides additional evidence to this possibility and showed that although BTEB1 gene transcription is regulated by estrogen, there is no parallel increase in BTEB1 protein levels with its mRNA. The present study is the first to show that Sp1 gene expression maybe regulated at the level of translation as well. However, previous studies showed that Sp1 is transcriptionally regulated since the concentration of the Sp1 protein changes in parallel with that of the mRNA (Saffer et al., 1990; Imataka et al., 1994), even in the case of Sp1 gene expression was upregulated by progesterone (Krikun et al., 2000). The mechanism underlying the present observation beyond this ubiquitously expressed transcription factor needs to be further clarified in future studies.

We have found that BTEB1 and Sp1 mRNA abundance are greater in uterine GE cells from D11 than from D14 pregnancy uterine endometrium. After treatment with E, BTEB1 and Sp1 mRNA levels in D11 GE cells were unchanged, whereas these were changed in D14 GE cells (Figure 3-4). We postulate that these responses to E are dependent on the corresponding basal mRNA levels. As shown in a previous study (Wang et al., 1997), GE cells express endogenous BTEB1 and Sp1mRNAs and proteins. GE cells isolated from D11 pregnant pig uterine endometrium have already been exposed to high concentrations of E since the embryos make copious mounts of E at the time. Perhaps, BTEB1 and Sp1 mRNA expression are already maximal at the time. On D14, when embryonic E has declined, BTEB1 and Sp1 levels are correspondingly low, hence treatment with E resulted in a detectable increase. In summary, it appears that E can upregulate BTEB1 and Sp1 mRNAs, although modestly. Thus, BTEB1 and Sp1 gene expression are regulated not only in a cell-type specific manner, but also in pregnancy

stage-specific manner that is related to the tissue steroid hormonal context. This result is in keeping with previous observations that BTEB1 mRNA levels in the cerebral cortex exhibit developmental regulation and thyroid hormone dependence, as BTEB1 mRNA exhibits a distinct elevation in expression that parallels developmentally-dependent increases in plasma T3 (Denver et al., 1999). Although BTEB1 protein levels did not change in consist with those of its mRNA in the uterine endometrium, it may change in other tissues with distinct translation machinery.

Figures 3-2 and 3-3 confirmed the E- and P- responsiveness of the GE cells used in this study. As expected, E induced PR gene transcription in D11 and D14 pregnancy GE cells, and UF gene transcription had a tendency ($p=0.09$) to be increased by P in D11 GE cells. However, the effects were relatively small; Indeed, P had no effect on UF gene expression in D14 pregnancy GE cells. The reasons for such small changes are unknown but may include the following: 1) The GE cells were cultured for several days before they were treated with steroid hormones. During this time, cells may have lost a proportion of those receptors that mediate specific responses to these hormones stimuli. 2) For the UF gene, there may not have been enough PR in the cells for exogenous P stimulation due to decreased estrogen concentration at D14 relative to D11 pregnancy GE cells. 3) GE cells at D14 of pregnancy have been exposed to high E at D11 and D12 of pregnancy. Thus, this response to E maybe distinct from those cells that have not been otherwise exposed to high levels of E. Many proteins involved in the regulation of transcription may be affected by E stimulation, and within the three days period between D11 and D14 of pregnancy, significant changes can occur.

As previous studies have demonstrated, BTEB1 and Sp1 can regulate the expression of a number of pregnancy-associated endometrial genes *in vitro*, such as UF and SLPI (Simmen et al., 1999; Simmen et al., 2000; Zhang et al., 2001). From the present study, we demonstrated that in endometrial GE cells, BTEB1 and Sp1 protein levels do not change in response to the steroid hormones, E and P, in contrast to their mRNAs although the response was very modest in the latter. There has been much evidence to support the existence of functional interactions between the transcription factors BTEB1, Sp1, PR and ER. These include P mediation of PR interaction with Sp1 and CBP/p300 (Owen et al., 1998), E recruitment of Sp1 to GC-boxes and interaction with ER as mediated by CBP/p300 (Scholz et al., 1998), E induction of Sp1 activity (Kleinert et al., 1998), and formation of ER/Sp1 complex (Rishi et al.,1995; Porter et al., 1996; Wang et al., 1998; Duan et al., 1999; Qin et al., 1999; Xie et al.,1999). These results indicate functional relationships between BTEB1 and/or Sp1 with the steroid hormones E and P and their respective receptors. During pregnancy, PR expression declines in uterine endometrium in several species, such as in the cow (Kimmins and MacLaren, 2001), horse (McDowell et al., 1999), rat (Ogle et al., 1998), rabbit (Gutierrez-Sagal et al., 1993), and in secretory glandular epithelial cells of the human (Perrot-Applanat et al., 1994). Therefore, although changes in the protein abundance of BTEB1 and Sp1 may not occur in the endometrium during pregnancy, the extent of the interactions between those factors and the steroid hormone receptors may change, resulting in the differential regulation of pregnancy-associated gene expression.

CHAPTER 4
INTERACTION BETWEEN BTEB1 AND PROGESTERONE RECEPTOR MEDIATES PROGESTERONE-RESPONSIVE GENE EXPRESSION IN ENDOMETRIAL EPITHELIAL CELLS

Introduction

During mammalian pregnancy, progesterone (P) exerts a critical influence over the status of the uterine endometrium in preparation for implantation and subsequent embryo development. In this tissue, P in concert with estrogen (E) and uterine-associated growth factors, coordinates cellular proliferation, differentiation, and apoptosis in a temporal- and spatial-specific manner, by binding to its nuclear receptor PR (Tsai and O'Malley, 1994), which is expressed and regulated distinctly in the epithelial and stromal compartments of the uterus (Tibbetts et al., 1998; Kurita et al., 2000). Recent studies on the molecular biology of the PR in rodents and humans indicate that two PR isoforms, namely PR-A (94 kDa) and PR-B (114 kDa), mediate the effects of P (Horwitz and Alexander, 1983). These isoforms are transcribed from a single gene using distinct estrogen-inducible promoters and differ only by the presence of the most amino-terminal 164 amino acids in PR-B, relative to PR-A (Kastner et al., 1990). Detailed structure/function studies on these PR isoforms indicate that PR-B in all cellular contexts *in vitro*, functions as a ligand-dependent transactivator of P-responsive genes in contrast to PR-A, which in some contexts, acts as a ligand-dependent transcriptional repressor of PR-B as well as of other steroid hormone receptors (Vegeto et al., 1993; McDonnell and Goldman, 1994; McDonnell et al., 1994; Giangrande et al., 1997), especially when

expressed in significant excess over PR-B (McGovern and Clarke, 1999). The latter observation has been attributed in part, to the distinct conformations achieved by each isoform in the presence of ligand, resulting in their differential ability to bind co-activators or co-repressors of transcription (Giangrande et al., 2000) as well as in their distinct susceptibility for phosphorylation on specific serine residues (Clemm et al., 2000).

Recent studies using mouse mutants in which either PR-A or PR-B expression has been selectively ablated, indicate that each PR isoform mediates distinct physiological responses to P (Lydon et al., 1995; Mulac-Jericevic et al., 2000). Indeed, a subset of P-target genes was identified to be transactivated selectively by PR-B, implying that the 164 amino acid region in the N-terminal portion of the protein mediates its specific interactions with numerous nuclear factors essential for transcriptional activity (Schwerk et al., 1995; Bamberger et al., 1996). One recently described PR-interacting partner is the GC-box-binding transcription factor Sp1, a seemingly ubiquitous nuclear protein and a member of the KLF family (Kadonaga et al., 1987; Turner and Crossley, 1999). In that study, Sp1 was shown to mediate the transactivation of the cyclin-dependent kinase inhibitor p21 gene promoter, which lacks consensus P-response elements, by forming a functional multi-protein complex with PR/P and another transcription activator CBP/p300 (Owen et al., 1998). Sp1 has also been implicated in the regulation of estrogen-, retinoic acid- and androgen-responsive genes via the formation of similar complexes (Qin et al., 1999; Husmann et al., 2000; Petz and Nardulli, 2000; Lu et al., 2000). Interestingly, Sp1 is generally considered a global, housekeeping transcription factor, whose expression is more constitutive relative to other family members (Philipsen

and Suske, 1999; Treichel et al., 2001). However, no evidence has yet been provided to indicate that other KLF family members, which now total at least 20 and whose individual expression displays more spatial-, temporal- and developmental-specificity than Sp1, can similarly mediate PR-dependent gene regulation in target cells.

The present study stems from previous work (Simmen et al., 1999; Simmen et al., 2000) in which we showed that the uterine endometrial expression of Basic Transcription Element Binding (BTEB1) protein, a KLF-family member (Turner and Crossley, 1999; Philipsen and Suske, 1999), is associated with transactivation of the gene encoding the P-dependent uterine endometrial protein uteroferrin (UF). In those studies, we raised the possibility of functional interactions between BTEB1 and PR/P, based on the model of Sp1/PR-P interactions, since (1) like that of Sp1, endometrial BTEB1 expression is constitutive and preferential to epithelial cells (Simmen et al., 2000; Wang et al., 1997); (2) Sp1 regulation of UF promoter activity *in vitro* was altered by co-expression of BTEB1 (Simmen et al., 2000); and (3) Sp1 and BTEB1 proteins are temporally co-expressed with PR in the early pregnancy endometrium (Simmen et al., 1988; Wang et al., 1997; Simmen et al., 1999; Simmen et al., 2000). The present study shows that distinct cellular levels of BTEB1 alter the P-sensitivity of certain gene promoter to transactivation by PR-B. We further show the effect of BTEB1 on the UF promoter activity when both PR-A and PR-B co-exist in target cells. Finally, the interaction between PR-B and BTEB3, the closest BTEB1 family member, is also investigated in this study. Results implicate two Sp/Krüppel family members, BTEB1 and BTEB3, as PR-interacting partners and suggest a novel mechanism by which P regulates expression of its target genes in epithelial cells.

Materials and Methods

Reagents were obtained as follows: Cell culture media from GIBCO-BRL (Grand Island, NY). All molecular biology-grade chemicals and solvents, when not specified, were purchased from Fisher Scientific (Pittsburgh, PA).

Cell Culture

The human endometrial carcinoma cell line Hec-1-A (gift from the late Dr. PG Satyaswaroop, The Hershey Medical Center, Hershey, PA) and stable transfectants derived from these cells which express high and low levels of BTEB1 (designated 4S and 2As cell lines, respectively) (Zhang et al., 2001) were cultured as described previously (Simmen et al., 1999). Cells were incubated with McCoy's 5A medium (GIBCO-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS) at 37°C under 95%O_2/5%CO_2 condition. Cell medium was changed every other day for cell propagation.

Transient Transfections and Reporter Gene Assays

Twenty-four hours before transfection, cells were plated in six-well plates at a density of 6 x 10^5 cells per well. Cells were transfected by the polybrene method with UF promoter-Luciferase (UF-Luc) reporter plasmid (10μg), insulin growth factor binding protein-2 (IGFBP-2) promoter (-1397 and –305)-Luciferase (IGFBP-2-1397-Luc, IGFBP-2-305-Luc) reporter plasmids (10μg, described in Badinga et al., 1998) in the presence or absence of expression vector for the full-length rat PR-B or corresponding empty vectors (1μg each), following previously described protocols (Simmen et al., 1999). A synthetic progestin (R5020; 10 or 100 nM; NEN Life Science Products Inc., Boston, MA) was added immediately after transfection. Luciferase assays of resultant

cell lysates were performed 48 h after transfection, following the manufacturer's instructions (Promega Corp., Madison, WI). Luciferase activity was normalized to cellular extract protein amounts, which were determined by the Bradford dye-binding procedure (Bradford 1976).

In a number of experiments, Hec-1-A cells were co-transfected with UF-Luc (5μg) reporter construct and combinations of expression vectors for the full-length rat BTEB1, human BTEB3 (also called RFLAT-1, generously provided by Drs. Alan Krensky and An Song of Stanford University, CA), human PR-A, human PR-B (both generously provided by Dr. Pierre Chambon) or their corresponding empty vectors (0.5μg for every construct). The cell culture and transfection methods followed previously described protocols from our laboratories (Simmen et al., 1999; Zhang et al., 2001).

Monkey kidney Cos-1 cells (American Tissue Type Culture, Manassas, Va) were also used for transfection assays. Cells were propagated in DMEM containing 10% fetal bovine serum (FBS) and grown in six-well plates to 60-70% confluence. Co-transfections were carried out with the chloramphenicol acetyltransferase (CAT) reporter gene linked to mouse mammary tumor virus-long terminal repeat (MMTV-LTR) sequences representing -631 to +125 nt of the 5' regulatory region of the MMTV gene (Cato et al., 1986) (also a gift of Dr. Pierre Chambon) and expression constructs (pCDNA3-BTEB and/or pCMV5-PR-B) or empty vectors (pCDNA3, PCMV5) in the presence or absence of R5020 (50 nM) by the lipofectamine method (Zhang et al., 2001). Cells were harvested 24 h later and assayed for CAT activity using the CAT ELISA kit (Roche Diagnostics Corp., Indianapolis, IN). The CAT activity was measured by absorbance at

405 nm. The CAT concentration (pg/ml) corresponding to an absorbance reading was calculated using a standard calibration curve and normalized to cellular protein content. Transfection data are presented as Least-squares means (LSM) ± SEM from three independent experiments, where each experiment was performed in triplicate.

Statistical Analysis

All numerical data were compared to appropriate controls as indicated for each experiment and analyzed using ANOVA following the General Linear Models Procedure of the Statistical Analysis System (SAS)(SAS 1988). Comparisons between groups were analyzed using predicted differences (pdiff) of the Least-squares means. Luc and CAT values were normalized to the protein concentration of the cell extract. The statistical model included treatment and experiment and only preplanned comparisons were made. Treatment means were considered significantly different at $p<0.05$.

Results

Sequence Analysis of Progesterone Response Elements (PREs) and GC-boxes in UF and IGFBP-2 Promoter-reporter Constructs

The porcine UF-Luc reporter plasmid contains 1143 bp of UF promoter region and the porcine IGFBP-2-1397-Luc and IGFBP-2-305-Luc reporter plasmids contain 1397 bp and 305 bp of IGFBP-2 gene 5'-flanking region, respectively (Reed et al., 1996; Song et al., 1996; Badinga et al., 1998). UF promoter sequence (retrieved from Genbank, Accession Number L23176) and the 1308 bp of IGFBP-2 promoter sequence starting from the transcriptional initiation site (Song et al., 1996) were analyzed using programs available at the web site www.motif.genome.ad.jp for nuclear sequence motifs from the motif library TRANSFAC. Results showed that there are four GC-boxes in UF-Luc, three GC-boxes in IGFBP-2-1397-Luc, and two in the IGFBP-2-305-Luc construct (Figure 4-1,

4-2, underlined bold letters). GC-boxes in the UF-Luc construct are located in the distal and middle portion of the promoter region (transcription initiation site designated as +1 in figures), whereas there are two GC-boxes very close to the +1 site in the IGFBP-2 constructs. There are two closely located non-consensus half PREs (Figure 4-1, Italic bold letters) in the UF-Luc construct (Lamian et al., 1993). However, IGFBP-2-1397-Luc contains only a half consensus PRE (Strahle et al., 1987), TGTTCT from –709 to –703 site (demarcated from the +1 site) (Figure 4-2, Italic bold letters indicating PRE). However, the construct IGFBP-2-305-Luc does not contain any consensus or half PRE.

Transactivation of Porcine UF and IGFBP-2 Gene Transcription by PR-B ± P in High and Low BTEB1-expressing Hec-1-A Cells

These laboratories previously generated Hec-1-A sublines by stable transfection with BTEB1 expression constructs in the sense and antisense orientations that have higher (4S) or lower (2As) BTEB1 expression levels than corresponding parent (untransfected) cells (Zhang et al., 2001). Evaluation of the promoter activity of the transiently transfected UF-Luc reporter construct in the generated stable sublines indicated increased stimulation of basal Luc activity in higher BTEB1-expressing cells (Zhang et al., 2001), confirming previous observations that demonstrated transactivation of the UF gene promoter by BTEB1 (Simmen et al., 1999). We have also previously ascertained that UF gene expression is induced by P (Simmen et al., 1989), at least in part at the level of its promoter (Simmen et al., 1999; Lamian et al., 1993). To examine the effect of BTEB1 on PR-B-mediated P stimulation of the UF gene promoter, PR-B expression construct or empty vector (pCMV5) and UF-Luc reporter construct were transiently co- tranfected in 4S and 2As lines, in the presence or absence of the synthetic

Figure 4-1. Porcine uteroferrin (UF) promoter (-1143) 5'flanking region. The sequence is retrieved from Genbank (Accession number L23176), and analyzed using web site www.motif.genome.ad.jp for nuclear sequence motifs from the motif library TRANSFAC. The GC-boxes in the sequence are designated with the bold underlined letters, and the non-consensus half PREs, which were identified in a previous study (Lamian et al., 1993), are designated by bold italic letters. The bold and capitalized letters indicate the transcription initiation site +1, and the end of this promoter sequence (-1143) in the reporter construct UF-Luc.

acagtcttggagctgagcagagccaggattc**T**gagatcacctgggccgcccggggccct**g**
-1143
gggcagggggcgggagggccgccccgacggcgagcgccctgcact**cctaca**gcgtggctcc
GC-box **PRE**
cgcggccgctcc***tggcct***tggccctgggccctgccgactgcaggtgagacaacccgcccg
PRE
gcgggtgtagaacccgccagaatccgccacccggaggaggggtctccgcctgccctggt

gacccactttctgtttgtcggggatgcgggagccagagggacagccagccggtgtcctc

agccgtgaatgaggggctcacatcatgcaagagaccgtagggagccatagtgagtgtgc

aggggacaaccagggccgggaggagggagcag**gaggggaggggcag**gtcagacagccctg
GC-box
tggtgcagcccttc**ccccagcccc**aagaggctgagggtgtgagagtcaaaagtctgtggt
GC-box
cacagggtagtcaccagctgcctcctgcctgggttcctcctgcaggaatgggggtcaggc

aggacttcctggaaaaagggagccccagctgttt**gggggaggggag**gtgaccctgggaca
GC-box
ctggctgggatgttgtcaaacacagctcacacacaggacacacagctcagctgagaaaca

caacagagacacacaacttgggcacaaaacaggcacacacaagtcgccttccccctaca

cacagttccacttgaggactcaggtgcacgtcacagacatgccatgcaggccttgccagc

tgagtatacacagccactggaacactggctcccaggctcacaaccaacatgtgactgagg

caggcacgcttggctacacgcagaaaacagctcagacacacccagacacaggcagacaaa

caccacctctgagaacaccgacacacaggaggtgcctggcccgggcattcggacacagct

gcctacacagcacacacgggccccgagacacgaactgctcctgtgggcagacgcaggggc

tgtggggcaggcgcagctgcactggtgcaccagacagccacctcaaatcccgtgtccagc

gctggggaactaactccaaggctcacatgacccgaggggagggcttttggaacagttggc

ggagaaactgcatcatcctcactaatgatccatttccg**c**
+1

UF Promoter

Figure 4-2. Porcine IGFBP-2 promoter (-1308) 5'flanking region. The sequence (Song et al., 1996) was analyzed using the web site www.motif.genome.ad.jp for nuclear sequence motifs from the motif library TRANSFAC. The GC-boxes in the sequence are designated with bold underlined letters, and the consensus half PRE (Strahle et al., 1987) is designated by bold italic letters. The bold and capitalized letters indicate the transcription initiation site +1, the end of the promoter sequence (-305) in the reporter construct IGFBP-2-305-Luc, and the end of promoter sequence (-1308) in the reporter construct IGFBP-2-1397-Luc (Song et al., 1996).

Agcttcaatttcaattctgatcccacccctgaaaccccatcacctttctatctaaatg
-1308
cgtgtctcctcctcaaagcctctggcaatctgttgttggttcattacaccaaactctcc

cccaaatggttgttcttgtccatttttgaagatcagaaacttgccctcctgggacatcc

agctcaccttcaaagccaggtagccacctgacaaaagctgcttcagtaaagtcttgaag

ttggaaagagctggagtgcagggccctggcgggggggaagggtggcacagaggtattgag

aaattatacctggtctggaggcagcttaatggcccacccccaccaagagaatctctct

gacaaggatctttgttagggtcacaacccagggagatgggtgataattaaagtggggtc

cagcctggaatttctgggagctgggaacagcttggatcaatgattcagtaaatcaaaca

gaagaataaagggaggccattcttgttcacctccatttactgaggagagctccaaagga

cctaacctcagggcattctcctacacctggaaagctgatctgacctctttaagatccct

gaagcagggt***gtttct***ggggtctatcctgcagcaaatgtctggagatctgtggg**tggggg**
PRE **GC-box**
ccgggcccccaagagattaatttctggggagggagcagactaggggagaggaagggag

caggtttctcagtgtccagaaactgcatttcaggtacttacatgttaccagatgctccc

agggttgggaaattgcaatggtccctaagaggggctattcagggtacgctggtctagag

gatgtgaacagcctccagactcctaagtcattgtcccaggcagcagtgagtgaaaaccc

gaggatggaggcagttgcagggagaggactgaaacctacttgaaggtcatgcctgacgt

cctttgccggccacatgggaagcgcgcaaacgaagtgcttccgaattgaaccgaaaac**A**
-305
ggagacaaaagcacccactcttgtccagtcccgcgccaatcttactcaaaccccaaat

tattctcatcctccgggagtcagggagcccggggtagaagtgcgtggagggacacagac

cggagggagttaggggacccccgcagcaggcgaggaagtggggatgggcgagtgggcg

tgcgcgcacgtagaggcccgcgcgcgggagtgtcgggggaaggggtggtctccaaa**ag**

ggggaggggagaaggc**aggggggcggggag**aagcagggccctttaggacccggctgcggc
GC-box **GC-box**
ggcgggggag**G**agaaagaagccgaggaggcggctcccgcgctcgaagggccgtgccacc
+1

IGFBP-2 Promoter

progestin R5020. In the absence of its ligand, PR-B decreased basal UF-promoter activity in both lines (Figure 4-3). The PR-B inhibition of UF promoter activity was reversed by the addition of R5020, although the fold-induction achieved over basal activity was dependent on BTEB1 expression levels in transfected cells. Whereas ligand-bound PR-B elevated Luc activity to only slightly higher ($p<0.05$) than basal levels in low expressing BTEB cells, its effect was greater in high BTEB1-expressing cells, where a 4.5-fold increase over basal activity was observed. Interestingly, the ligand-dependent increase in Luc activity by PR-B was only achieved at higher R5020 concentration (100 nM but not 10 nM) in low BTEB1 expressing lines, but was comparable at 10 and 100 nM R5020 in high BTEB1 expressing lines. Thus, the relative amount of BTEB1 in P-responsive cells appears to influence the sensitivity of the target gene promoter to transactivation by ligand-bound PR-B.

To test the effects of BTEB1 on other gene promoters in the presence of PR-B, IGFBP-2 promoter reporter constructs, IGFBP-2-1397-Luc and IGFBP-2-305-Luc, as well as PR-B expression construct or empty vector (pCMV5), were transient transfected in the Hec-1-A sublines that have higher (4S) or lower (2As) BTEB1 expression levels. Both constructs showed increased stimulation of basal Luc activity in higher BTEB1-expressing cells, and confirmed the transactivition of BTEB1 on the two IGFBP-2 promoter constructs, which contain three or two GC-boxes respectively. PR-B decreased basal IGFBP-2-1397 promoter activity in the absence of its ligand and decreased this further ($p<0.05$) in the presence of its ligand, and the effects of PR-B were the same between the two sublines (2As and 4S, Figure 4-4). In the IGFBP-2-305 promoter reporter construct, PR-B increased basal Luc activity in the absence of ligand in both

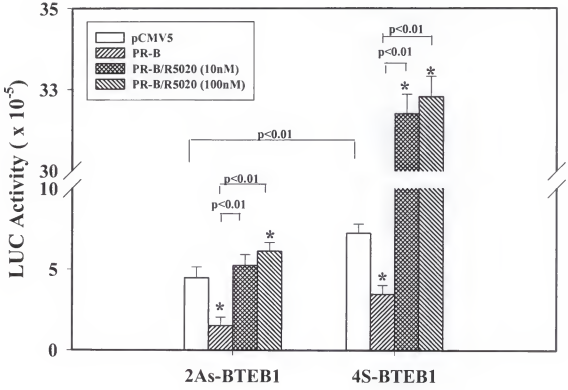


Figure 4-3. Effect of BTEB1 on the transactivation of UF gene promoter by PR-B in the presence and absence of R5020. Stably transfected Hec-1-A cell lines expressing high (4S-BTEB) and low (2As-BTEB) levels of BTEB1 were co-transfected with empty vector (pCMV5) or pCMV5-PR-B expression vector and UF-Luc reporter construct, in the presence or absence of R5020 (10 or 100 nM). Results (Luciferase activity normalized to cellular protein content) are presented as Least-squares mean (LSM) ± SEM of three independent experiments, each performed in triplicate. * denotes statistical significance of $P<0.05$, compared with basal activity (pCMV5 alone), by ANOVA. Significant differences between treatment groups (indicated by brackets) were also identified by ANOVA.

lines. However, this increase was reversed by the presence of ligand, R5020, and the inhibition of IGFBP-2-305 promoter activity by PR-B/P in two lines was the same (Figure 4-5). Since the IGFBP-2-1397-Luc construct contains additional IGFBP-2 promoter-flanking sequence and an extra half PRE site, different effects of PR-B on this promoter are anticipated. Although the promoter activities of both constructs, which contain multiple GC-boxes, were enhanced by BTEB1, this nuclear protein did not influence the sensitivity of the target gene promoter to transactivation by ligand-bound PR-B.

The Effect of BTEB1 on the Transactivation of the MMTV Promoter by PR-B±P

The experiments described above demonstrated that BTEB1 affects the activity of ligand-bound PR-B depending on gene promoter context in uterine endometrial epithelial carcinoma cell lines. Since the UF promoter contains two closely located non-consensus half-site PREs rather than only one half-site PRE as in the IGFBP-2-1397 promoter, it seems likely that PR-B dimer formation is important for its interaction with BTEB1. Other experiments, including co-immunoprecipitation, mammalian two-hybrid system, and gel retardation assays, were used to demonstrate the physical interaction between PR-B and BTEB1 (Zhang et al., 2002). To determine if the ligand-bound PR dimer forms a functional complex with BTEB1, independent of BTEB1 DNA-binding activity, transient co-transfection experiments were carried out in Cos-1 cells with expression constructs for BTEB1and/or PR-B in the presence or absence of added R5020, using a reporter gene MMTV-LTR-CAT, which contains recognition sequences for PR but not BTEB1 (Cato et al., 1986). As anticipated, BTEB1 had no effect on MMTV-LTR promoter activity, while PR increased this gene's transcriptional activity in a P-dependent manner (Figure 4-6). Moreover, the combination of ligand-bound PR-B and BTEB1 increased MMTV-

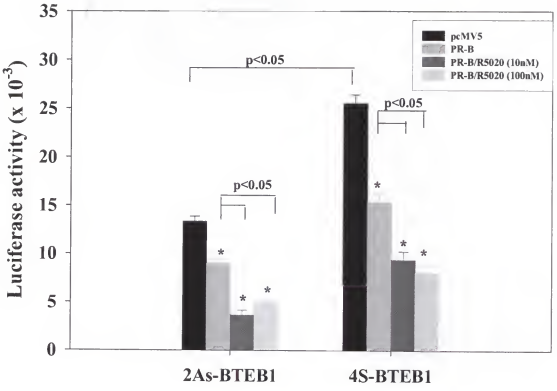


Figure 4-4. Effect of BTEB1 on the transactivation of IGFBP-2 gene promoter –1397 construct by PR-B in the presence and absence of R5020. Stably transfected Hec-1-A cell lines expressing high (4S-BTEB) and low (2As-BTEB) levels of BTEB1 were co-transfected with empty vector (pCMV5) or pCMV5-PR-B expression vector and IGFBP-2-1397-Luc reporter construct, in the presence or absence of R5020 (10 or 100 nM). Results (Luciferase activity normalized to cellular protein content) are presented as Least-squares mean (LSM) ± SEM of three independent experiments, each performed in triplicate. * denotes statistical significance of $P<0.05$, compared with basal activity (pCMV5 alone), by ANOVA. Significant differences between treatment groups (indicated by brackets) were also identified by ANOVA.

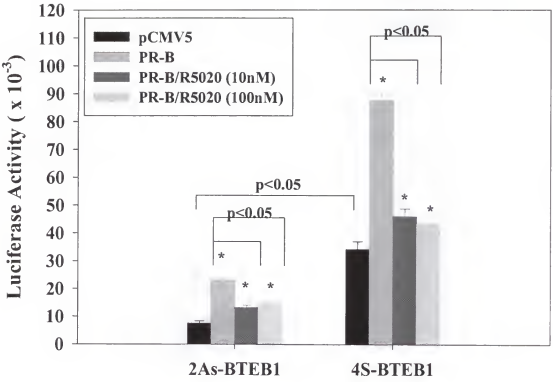


Figure 4-5. Effect of BTEB1 on the transactivation of IGFBP-2 gene promoter –305 construct by PR-B in the presence and absence of R5020. Stably transfected Hec-1-A cell lines expressing high (4S-BTEB) and low (2As-BTEB) levels of BTEB1 were co-transfected with empty vector (pCMV5) or pCMV5-PR-B expression vector and IGFBP-2-305-Luc reporter construct, in the presence or absence of R5020 (10 or 100 nM). Results (Luciferase activity normalized to cellular protein content) are presented as Least-squares mean (LSM) ± SEM of three independent experiments, each performed in triplicate. * denotes statistical significance of $P<0.05$, compared with basal activity (pCMV5 alone), by ANOVA. Significant differences between treatment groups (indicated by brackets) were also identified by ANOVA.

LTR promoter activity over that achieved with PR-B dimer alone, confirming that a functional interaction between PR-dimer and BTEB1 can occur without BTEB1 binding to its canonical GC-rich DNA sequence.

Effects of PR-A and PR-B on UF Promoter Activity with and without BTEB1

To investigate the relationship between BTEB1, PR-A, and PR-B, on UF gene transcription, Hec-1-A cells were transiently co-transfected with UF-Luc reporter construct and expression constructs for BTEB1, human PR-A and human PR-B, alone or in combination, in the absence or presence of R5020 (100nM). As shown previously, BTEB1 itself increased basal Luc activity, PR-B without ligand decreased the basal Luc activity, and the inhibition of PR-B on UF promoter activity was reversed in the presence of R5020. When co-transfected with both PR-B and BTEB1 in the presence of R5020, UF promoter activity was further increased from that of ligand-bound PR-B alone. Similar to PR-B, PR-A decreased ($p<0.05$) the basal Luc activity in the absence of ligand. However, in contrast to PR-B, the basal UF promoter activity was not increased by ligand-bound PR-A nor by BTEB1 when co-transfected with PR-A in the presence of R5020 (Figure 4-7). Basal Luc activity was decreased ($p<0.01$) when PR-A and PR-B were co-transfected. However, although the inhibition was reversed when the ligand was added, consistent with that shown for liganded PR-B, the promoter activity achieved in the presence of both isoforms was less than that for PR-B alone with ligand ($p<0.05$). When PR-A, PR-B and BTEB1 expression plasmids were co-transfected, in the absence of ligand, the Luc activity was not statistically difference from the basal levels. Interestingly, in the presence of ligand, the Luc activity increased, but at a lesser magnitude that obtained with the combination of BTEB1 and ligand-bond PR-B. Moreover, the increase in Luc activity above basal was much less with the combination

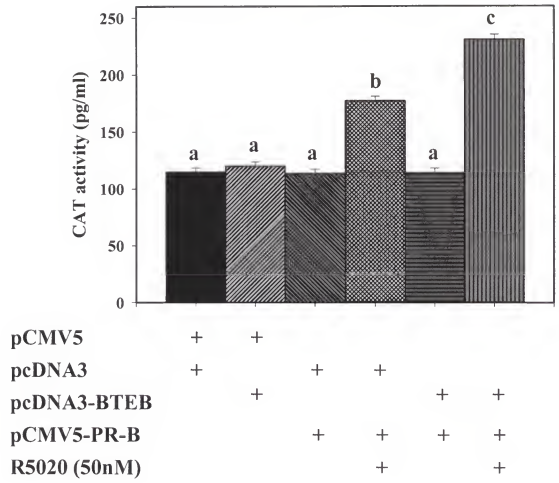


Figure 4-6. Effect of BTEB1 on PR-B/P-mediated transactivation of the MMTV-LTR gene promoter. Cos-1 cells were transiently co-transfected with BTEB1 expression vector (pcDNA3-BTEB1; 0.5μg), PR-B expression vector (pCMV5-PR-B; 0.5μg) and/or corresponding empty vectors (pcDNA3, pCMV5; 0.5 μg each) and the MMTV-LTR CAT reporter construct (5μg), as described under Materials and Methods. Cells with added PR were incubated in the presence or absence of R5020 (50 nM). Results are presented as LSM ± SEM of three independent experiments, with each experiment performed in triplicate. Means without a common superscript significantly differ ($P<0.05$).

of PR-A and PR-B in the presence of R5020. Thus, BTEB1 has dual effects on PR transactivity for the target gene promoter depending on the isoform of PR expressed in target cells. On one hand, BTEB1 can synergistically increase the transactivation by PR-B + P of target gene promoters in the absence of PR-A; on the other hand, it can also inhibit the transactivation of target gene promoters in the presence of PRA-PR-B hetero-dimer.

Transactivation by BTEB3 of UF Promoter Activity in the Presence of PR-B±P

To extend the investigation on the functional relationships between PR-B and other Sp/Krüppel-like family members, BTEB3, also called RFLAT-1 and the closest family member to BTEB1, was co-transfected with the UF-Luc reporter construct and human PR-B expression construct in the absence or presence of R5020. Compared to BTEB1, BTEB3 itself did not transactivate the UF promoter (Figure 4-8). When co-transfected with BTEB1, BTEB3 did not show any additive nor synergistic effect on BTEB1's transactivity. However, BTEB3 increased Luc activity to the same extent as BTEB1 when it was co-transfected with PR-B in the presence of ligand. Thus, BTEB3 appears to have the same influence on PR-B transactivation of the UF promoter as BTEB1, and suggestive of likely similar mechanism utilized by BTEB1 and BTEB3 for interaction with PR-B.

Discussion

The purpose of this study was to further analyze the nature of and the molecular mechanisms underlying, putative functional interactions between PR and BTEB1 in the

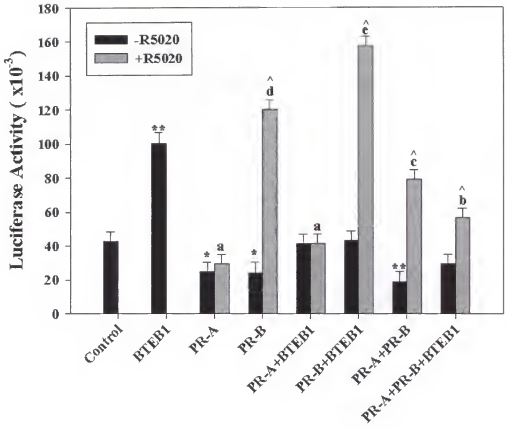


Figure 4-7. Effect of BTEB1 on PR-A and PR-B mediated transactivation of the UF gene promoter in the presence or absence of P. Normal Hec-1-A cells were transiently co-transfected with BTEB1 expression vector (pcDNA3-BTEB1; 0.5μg), human PR-A and/or human PR-B expression vector (pSG5-PR-A, pSG5-PR-B; 0.5μg) and/or corresponding empty vectors (pcDNA3, pSG5; 0.5 μg each) and the UF-Luc reporter construct (5μg), as described under Materials and Methods. Cells with added PRs were incubated in the presence or absence of R5020 (100 nM). Results are presented as LSM ± SEM of three independent experiments, with each experiment performed in triplicate. The means represented by black bars with one ($p<0.05$) or two ($p<0.01$) * differ from the control (UF-Luc with empty expression vectors only); the means represented with gray bars without a common superscript significantly differ ($p<0.05$); and the means represented by gray bars with a ^ differ from the means represented by black bars within the same group.

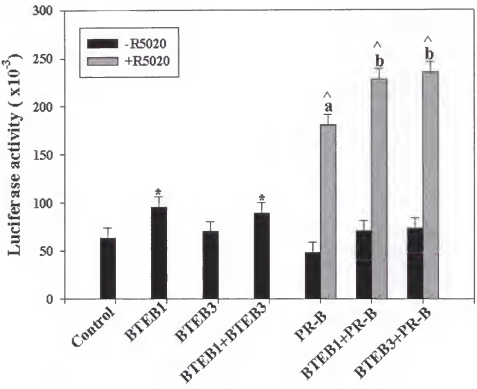


Figure 4-8. Effect of BTEB1 and BTEB3 on PR-B/P-mediated transactivation of the UF gene promoter. Normal Hec-1-A cells were transiently co-transfected with BTEB1 or BTEB3 expression vector (pcDNA3-BTEB1, pBK-CMV-BTEB3; 0.5μg), human PR-B expression vector (pSG5-PR-B; 0.5μg) and/or corresponding empty vectors (pcDNA3, pBK-CMV, pSG5; 0.5 μg each) and the UF-Luc reporter construct (5μg), as described under Materials and Methods. Cells with added PR-B were incubated in the presence or absence of R5020 (100 nM). Results are presented as LSM ± SEM of three independent experiments, with each experiment performed in triplicate. The means represented by black bars with * differ from the control (UF-Luc with empty expression vectors only); the means represented with gray bars without a common superscript significantly differ ($P<0.05$); and the means represented by gray bars with a ^ differ from the means represented by black bars within the same group.

transactivation of the gene encoding UF, as a paradigm for pregnancy-associated, endometrial epithelial-specific gene transcription. Previous studies have demonstrated the UF gene to be regulated at the level of its promoter by the steroid hormone progesterone (Simmen et al., 1989; Lamian et al., 1993; Simmen et al., 1999) as well as the GC-box binding transcription factors Sp1 and BTEB1 (Simmen et al., 1999; Simmen et al., 2000). Since Sp1 has been functionally linked to several steroid hormone receptors, including PR, in the transactivation of a number of genes, each of which exhibits specific spatial- and temporal patterns of expression (Owen et al., 1998; Qin et al., 1999; Husmann et al., 2000; Petz and Nardulli, 2000; Simmen et al., 2000), and since BTEB1 and Sp1 may mediate gene transcription by binding to the same recognition sequences within target genes (Sogawa et al., 1993b; Gonzalez et al., 1995), the possibility that BTEB1, similar to Sp1, interacts with PR to functionally regulate endometrial gene expression during pregnancy seemed likely. In the absence of any well-characterized mammalian epithelial cell line from uterine endometrium of pregnancy, two lines derived from human endometrial carcinoma Hec-1-A cells were used, which were engineered to express high (4S) and low (2As) levels of BTEB1, respectively (Zhang et al., 2001) and which were transiently co-transfected with PR-B, to mimic the status of the pregnancy endometrium. Using this *in vitro* model, the present study showed that the sensitivity of endometrial epithelial cells to PR-B-mediated P induction of UF gene promoter activity was influenced by the cellular expression levels of BTEB1. In particular, it was observed that maximal transactivation of basal UF promoter activity by PR-B (an increase of at least 4.5-fold) was achieved at 10-fold lower R5020 concentration in a high BTEB1 background than in a background of low BTEB1. Further

studies show that this effect of P is likely a consequence of direct interactions between BTEB1 and PR-B (data was published in Endocrinology Zhang et al., 2002). However, while unliganded PR and BTEB1 can form a complex *in vitro*, a productive BTEB1/PR-B interaction requires P. Similar experiments conducted on the IGFBP-2 promoter show that even though BTEB1 can functionally transactivate both promoters (-1397 and –305), it had no effect on PR-B's activity. These results suggest that a functional complex conferring P-dependent transactivation likely consists of BTEB1 in a ternary complex with the PR-B-dimer, although we cannot exclude the possibility that the PR-B-dimer/BTEB1 complex is part of a multi-complex unit that also includes other co-activators previously implicated in PR action (McKenna et al., 1999). The present study, also shows that PR-A, which usually acts as a transcription repressor by recruiting co-repressors (Giangrande et al., 2000), may also form a complex with PR-B-dimer and BTEB1, although the effect is a negative one. These results identify BTEB1 as a novel PR-interacting partner, and suggest that the transcriptional response of P-responsive genes to P in endometrial epithelial cells may involve BTEB1.

Present studies examined the functional interactions of PR and BTEB1 under no and high P conditions, the latter simulating the status of early pregnancy. In separate studies from our laboratories, the physical interactions of these nuclear proteins using nuclear extracts prepared from endometrium at different pregnancy days were also evaluated to provide biological context to this novel PR-transcriptional complex (Zhang et al., 2002). Two things are particularly worth noting from these combined studies. First, the observed reduction of basal promoter activity of the P-responsive UF gene in cells expressing both BTEB1 and PR-B in the absence of P was associated with P-independent

physical interactions between these nuclear proteins (Zhang et al., 2002). Second, the interaction of PR-B and BTEB1 within the context of the pregnancy endometrium was found to decrease at the late pregnancy stage (Zhang et al., 2002). These findings suggest that: 1) P is not required for PR-B-BTEB1 complex formation; 2) that the interactions of these proteins alone can modify the promoter activity of various genes whose regulatory regions, like that of UF, may contain recognition sequences for both; and 3) that changes in the amounts of BTEB1/PR-B complex formed with pregnancy status may underlie the temporal expression of distinct endometrial genes during this period. Indeed, since endometrial expression of BTEB1 protein appears constitutive across pregnancy (Wang et al., 1997), the observed decrease of the interaction of PR-B and BTEB1 within the context of the pregnancy endometrium may be a function of diminished BTEB1 activity and/or reduced concentrations of other proteins involved in the stabilization of the PR/BTEB complex. In this regard, numerous studies have now documented that the transactivation function of PR is governed by its ability to associate with transcriptional co-activators, and/or in its inability to recruit co-repressors (Owen et al., 1998; McKenna et al., 1999; Xu et al., 2000; Giangrande et al., 2000). Thus, BTEB1, in the presence of P, may enhance the biological activity of PR by facilitating the association of the PR-B-dimer with a number of co-activators, resulting in this receptor's increased interaction with the general transcription machinery. Confirmation of this hypothesis awaits the identification of specific coactivators whose association with PR is increased in the presence of BTEB1.

Previous studies from other laboratories (Imataka et al., 1992; Chen and Davis, 2000) as well as our own (Simmen et al., 1999) indicate that BTEB1 can act as a

bonafide DNA binding protein, via its own recognition sequence within target gene regulatory regions. Thus, the possibility that this nuclear protein may also function as an interacting partner with PR-B or PR-B/PR-A hetero-dimer as suggested from the present studies, raises questions as to how BTEB1 may perform the dual role of a transacting factor via binding to its specific cis-acting regulatory elements, and as a transcriptional coactivator, independent of DNA binding activity. The model presented in Figure 4-9 suggests a possible mechanism by which BTEB1 might perform these roles, which may be dependent on cell as well as gene context. In cells co-expressing PR and BTEB1, the transcription of gene promoters containing recognition sites for both BTEB1 and PR is predicted to be inhibited, relative to those containing BTEB1 recognition sequences alone, due to the likely formation of a non-productive, transcriptionally inactive BTEB1/PR complex. In the presence of P, however, the formation of the productive, transcriptionally active PR-dimer/BTEB1 complex is anticipated to enhance the activity of these gene promoters, above that predicted from the individual activities of BTEB1 and the PR-dimer, each of which bind to its respective recognition sequence. This model further presupposes that the formation of the PR/BTEB1 complex, under conditions of high or no P, is favored over that in which BTEB1 binds to its own recognition sequence. However, when PR-A isoform is co-expressed in P-responsive cells, it can interact with the PR-B-dimer/BTEB1 complex, recruits co-repressors, and mediates transcriptional repression overruling the transactivation mediated by the PR-B/BTEB1 complex. Together, these results suggest that BTEB1 can influence the direction of the transcriptional response of cells expressing endogenous PR, depending on the cellular PR-B and PR-A expression patterns as well as on physiological P status.

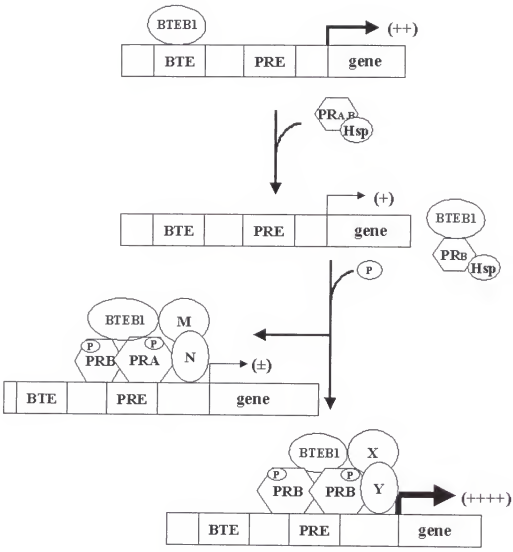
Besides Sp1 and BTEB1, results of the present study show that BTEB3 can functionally interact with PR-B. Although it binds to the classical BTE and GC-box with the same affinity as BTEB1 and Sp1 (Martin et al, 2000), BTEB3 behaves as a transcriptional repressor. This was demonstrated from studies in which the N terminus of BTEB3 was found to strongly repress transcription in a dose-dependent manner when tethered to DNA through the heterologous DNA-binding domain of GAL4 (Kaczynski et al. 2001). The repressor function of BTEB3 is due to its ability to interac with the co-repressor mSin3A and the histone deacetylase protein HDAC-1, as well as to its ability to compete with Sp1 for BTE binding (Kaczynski et al., 2001). In present study, BTEB3 was found to exert the same influence on ligand-bond PR-B transactivation function as BTEB1, although alone it had no effect on the transactivation by BTEB1 of UF gene promoter activity. This raises the possibility that BTEB3 and BTEB1 interact with PR-B via a common domain(s). The previously documented physical association of Sp1 with PR was shown to occur only in the presence of PR agonists (Owen et al., 1998). Since it may not be necessary for BTEB1 to bind to DNA when it is interacting with PR-B dimer (Zhang et al., 2002), the interaction may occur between the DNA-binding domain of BTEB1, BTEB3, or even Sp1, although further studies should be done to locate the interaction occurs.

Recently, we demonstrated that increased expression levels of BTEB1 in the endocarcinoma cell line Hec-1-A, which lacks PR, were correlated with increased proliferative status in response to serum-derived factors (Zhang et al., 2001). The increased proliferation seen in high BTEB1-expressing cells compared to those with low BTEB1 expression, was associated with increased traversal of the cell cycle, consistent

with these cells' enhanced expression of cyclin D1 and other growth-associated proteins (Zhang et al., 2001). Thus, BTEB1 can function to modulate cell growth, albeit the underlying mechanism is presently unclear. Interestingly, the findings reported herein that BTEB1 can productively partner with the PR-dimer, an inhibitor of cell proliferation through its transcriptional inhibition of growth-associated proteins (Clarke and Sutherland., 1990; Owen et al., 1998; Vazquez et al., 1999) suggests that BTEB1 manifests versatility in its growth regulatory activity, depending on cell context. These results underscore the importance of further analysis of BTEB1 interacting partners for understanding the molecular basis of endometrial growth regulation.

In conclusion, we demonstrate in the present study that BTEB1 likely plays an important role in PR-mediated gene transactivation by binding to PR. Given that BTEB1 can function as a positive growth regulator in some contexts, and that PR-B mediated P-transactivation has been shown to contribute to both P-dependent proliferative and anti-proliferative responses (Mulac-Jericevic et al., 2000; Clarke and Sutherland, 1990), a better understanding of the mechanisms underlying the formation of the PR-BTEB1 complex, and of the respective BTEB1 and PR domains involved in this interaction, could lead to the development of novel strategies for manipulating transcriptional events that control the balance of cellular proliferation and differentiation.

Figure 4-9. Model for BTEB1, PR-A and PR-B interactions within a P-responsive gene promoter. BTE and PRE represent DNA recognition sequences for BTEB1 and PR homo- or hetero-dimer, respectively. Heat shock proteins that bind unliganded PR are designated as Hsp. X and Y represent nuclear co-activators that interact with ligand-bound PR-B-dimer to form the activated transcriptional complex. In the unliganded state, the interaction of PR-B with BTEB1 can result in the inhibition of BTEB1 binding to BTE element. When P is present, the formation of a functional PR-B-dimer allows the subsequent formation of a complex involving BTEB1 and possibly other co-activators, likely leading to a stable and highly activated transcriptional state. When PR-A is present, PR-A, PR-B and BTEB1 can form an inhibitory complex by recruiting co-repressor M or N. (±), (+), (++) and (++++) represent low, basal, moderate, and high promoter activity, respectively.


BTEB1
(++)
BTE
PRE
gene
PRA,B
Hsp
(+)
BTE
PRE
gene
BTEB1
PRB
Hsp
P
BTEB1
M
P
P
PRB
PRA
N
(±)
BTE
PRE
gene
BTEB1
X
P
P
PRB
PRB
Y
(++++)
BTE
PRE
gene

CHAPTER 5
A PREGNANCY-ASSOCIATED, BTEB1 DOWNSTREAM GENE PRODUCT, SECRETORY LEUKOCYTE PROTEASE INHIBITOR, MEDIATES PROLIFERATION OF HUMAN ENDOMETRIAL GLANDULAR EPITHELIAL CELLS BY POSITIVE AND NEGATIVE REGULATION OF GROWTH-ASSOCIATED GENES

Introduction

Secretory leukocyte protease inhibitor (SLPI), also known in the literature as anti-leukoprotease inhibitor (ALP), human mucus proteinase inhibitor, and human seminal plasma inhibitor (HUSI-1), is a 12kDa member of the chelonianin class of serine protease inhibitors (referred to as serpins), which also includes SKALP/elafin (Potempa et al., 1994; Francart et al., 1997). It is comprised of two homologous cysteine-rich domains with inhibitory activities against chymotrypsin, trypsin, elastase, and cathepsin G (Thompson and Ohlsson, 1986; Stetler et al., 1986). SLPI is expressed primarily in secretory/glandular epithelial cells of a variety of human tissues including the bronchi, intestine, skin, breast, pancreas, male and female genital tracts, and kidney (De Water et al., 1986; Si-Tahar et al., 2000; Wiedow et al., 1998; Semba et al., 1999; Nystrom et al., 1999; Ohlsson et al., 1995; King et al., 2000; Heinzel et al.,1986; Helmig et al., 1995; Ohlsson et al., 2001). Its relative absence in serum, except in cases of pathological conditions (Maruyama et al., 1998; Grobmeyer et al., 2000), and its localization to extracellular matrix and sub-cellular sites not readily accessible to larger mol wt protease inhibitors such as alpha-1-anti-trypsin (Rice and Weiss, 1990), suggests autocrine/paracrine modes of action. In addition to its anti-protease activity, SLPI

functions as an anti-inflammatory, anti-bacterial, anti-fungal, and anti-viral (HIV) agent (Lentsch et al., 1999; Song et al., 1999; Gipson et al., 1999; Hiemstra et al., 1996; Tomee et al., 1997; Wahl et al., 1997). Further, SLPI has been shown to regulate intracellular enzyme synthesis, suppress matrix metalloproteinase production and activity, mediate normal wound healing, prevent scar formation, and augment fertility (Zhang et al., 1997; Ashcroft et al., 2000; Sumi et al., 2000; Moriyama et al., 1998). The higher uterine endometrial expression of SLPI during pregnancy in species with the epitheliochorial, as opposed to the haemochorial type of placentation, suggests an important role for this protein in the maintenance of an intact utero-placental interface (Badinga et al., 1994). Its relevance to the regulation of pregnancy-associated events is further bolstered by the recent findings of elevated SLPI expression in endometriotic tissues and corresponding peritoneal fluids (Suzumori et al., 1999), in amniotic fluids during the onset of labor (Denison et al., 1999), and in human fetal membranes and cervical mucus in normal pregnancy (Helmig et al., 1995).

A possible role for SLPI in the control of cell proliferation was initially gleaned from the high levels of SLPI found in epithelial carcinomas of numerous tissues (Garver et al., 1994; Robertson et al., 1998). Subsequently, SLPI was shown to support the growth of human hematopoietic progenitor cells in serum-free medium *in vitro* (Goselink et al., 1996), and to increase the production of hepatocyte growth factor in human lung fibroblasts (Kikuchi et al., 2000). Previous studies in Dr. Rosalia Simmen's lab demonstrated that SLPI, added at levels comparable to those found in uterine luminal fluids *in vivo*, increased DNA synthesis in primary cultures of glandular epithelial cells isolated from pregnant pig uterine endometrium (Badinga et al., 1999).

Moreover, clonal lines of the human endometrial epithelial cell line Hec-1-A over-expressing the transcription factor BTEB1 and consequently, exhibiting higher proliferative potential as well as increased expression of a number of cell cycle-associated genes, have augmented SLPI mRNA and protein levels, relative to slowly growing clonal lines with lower BTEB1gene expression (Zhang et al., 2001). Although the increased proliferative responsiveness to serum of higher-expressing BTEB1 lines has now been partially elucidated by the identification of BTEB1 gene targets and by the demonstration of BTEB1 transactivation of promoters for a number of growth-associated genes (Simmen et al., 2002), the mechanism underlying SLPI regulation of cell proliferation remains unresolved.

The present study demonstrates the regulation of SLPI promoter activity by BTEB1. Secondly, a well-differentiated human endometrial adenocarcinoma cell line Ishikawa (Nishida et al., 1985) was used to establish "SLPI knock-out" lines which stably express anti-sense SLPI mRNA. Using these clonal lines, directly linked cellular SLPI production and proliferation was established using DNA synthesis rate and cell number as parameters of growth. The study further shows that SLPI increases cyclin D1 gene expression and the transcriptional activation of its promoter, and identifies lysyl oxidase as an SLPI negatively regulated gene by the methodology of differential display-reverse transcriptase polymerase chain reaction. Further, this study demonstrates an inverse relationship between expression levels of SLPI and those of transforming growth factor (TGF)-β1 and insulin-like growth factor-binding protein (IGFBP-3), respectively. These results suggest that this novel function for SLPI, a BTEB1 downstream gene target, in epithelial cell proliferation is mechanistically distinct from its role in protease inhibition.

Materials and Methods

Reagents were obtained as follows: Restriction enzymes and Taq DNA polymerase from Boehringer Mannheim (Indianapolis, IN); nick-translation kit from Amersham Pharmacia Biotech Inc. (Piscataway, NJ); [α-^{32}P] deoxycytidine triphosphate (dCTP, 3000 Ci/mmol) and Biotrans nylon membranes (0.2μM) from ICN Radiochemicals (Irvine, CA); cell culture media from GIBCO-BRL/Invitrogen Corp. (Grand Island, NY); anti-cyclin D1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); anti-PCNA antibody from Roche Molecular Biochemicals (Indianapolis, IN); and pIND mammalian expression vector from Invitrogen Corporation (Carlsbad, CA). All molecular biology-grade chemicals and solvents, when not specified, were purchased from Fisher Scientific (Pittsburgh, PA).

Cell Culture and Treatments

The human endometrial carcinoma cell line Ishikawa (courtesy of Dr. Bruce Lessey, University of North Carolina, Chapel Hill) was routinely cultured in Minimal Essential Medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotic/antimycotic solution (ABAM) at 37°C in an atmosphere of 95%air /5% CO_2. Cells were grown in the same medium until they reached 90% confluence, at which time the medium was changed to serum-free. Twenty-four hours thereafter, cells received either recombinant human SLPI (100 ng/ml; R&D Systems, Inc., Minneapolis, MN) twice at 24 h intervals, or transforming growth factor (TGF)-β1 (10ng/ml; R&D Systems, Inc., Minneapolis, MN) added once. Cells were then collected 24 h after the last treatment. Control cells received only serum-free medium and were incubated for the same length of time as treated cells.

Generation of Stably Transfected Cell Lines

The full-length human SLPI cDNA (399 bp) was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using Ishikawa cell RNA as template, and forward (5'-ATGAAGTCCAGCGGCCTCTTC-3') and reverse (5'-TCAAGCTTTCACAGGGGAAA-3') primers that were synthesized based on the published sequence for human SLPI mRNA (Accession number AF114471). This fragment was cloned into the TOPO^{TM}TA cloning vector (Invitrogen), cleaved with *EcoR*I, and subcloned into pIND mammalian expression vector (Invitrogen). The sense and anti-sense orientations of the cloned fragments were determined by digestion with *EcoR*I and *Hind* III. Plasmid DNAs were prepared using the QIAGEN Plasmid Maxi Kit (Qiagen, Chatsworth, CA), sequenced to confirm the fidelity of the constructs (Interdisciplinary Center for Biotechnology Research, University of Florida), and used to transfect Ishikawa cells using Lipofectamine (GIBCO-BRL, Invitrogen Corp.), following the manufacturer's instructions. Transfected cells were cultured for 4-6 weeks in serum (10% FBS)-containing MEM supplemented with G418 (1mg/ml; Invitrogen), and surviving colonies were selected using cloning cylinders. Individual colonies were expanded in the same medium with added G418 (1mg/ml). Clonal lines were verified to express the anti-sense SLPI mRNA by RT-PCR analyses. This procedure utilized cDNAs synthesized from the total RNA of selected cell lines and primers (forward, 5'-CTCTGAATACTTTCAACAAGTTAC-3'; reverse, 5'-CTGTGGAAGGCTCTGGAAAG-3') located within the pIND vector and the anti-sense SLPI cDNA sequence, respectively. The presence of the amplified PCR product (451 bp)

was evaluated by electrophoresis in a 1% agarose gel and visualized using an ethidium bromide stain.

Cell Number and DNA Synthesis

Clonal lines stably expressing the empty vector alone (13pIND, control) or the anti-sense SLPI construct (8As) were seeded into 6-well tissue culture dishes at the same density ($1.2x10^5$ cells /well) in serum-containing medium 2 days before the start of the cell count (designated as Day -2). Cells were allowed to adhere to the plates for 24 h, and were induced to growth-arrest by changing the growth medium to serum-free. After 24 h, the cells received fresh medium containing 10% FBS. Every other day for a total of 8 days, the cells were counted under a microscope using a hemocytometer. The experiment was repeated three times, with each experiment carried out in triplicate wells.

To measure cellular DNA synthesis, cells were seeded at a density of $6x10^5$ cells/well in 6-well tissue culture dishes. At confluence, cells were incubated in serum-free medium for 24 h, and then transferred to serum-containing medium for another 24 h. At 4h before the end of the incubation period, cells were pulse-labeled with ^{3}H-thymidine (1 μCi/well). The processing of cells to quantify incorporated label followed previously described protocols (Simmen et al., 1988).

RNA Isolation and Northern Blot Analysis

Total cellular RNA (30 μg), prepared by the Trizol method, was electrophoresed in 1% agarose/formaldehyde gels in 1x MOPS (3-*N*-morpholino-propanesulfonic acid) buffer and blotted onto BioTrans nylon membranes (ICN Biotech, Irvine, CA) by downward capillary transfer using the TurboBlotting system (Schleicher and Schuell, Keene, NH). RNA was immobilized by UV-cross-linking for 90 sec followed by baking

in an 80°C oven for 25 min. Blots were pre-hybridized in ULTRAhyb ™ (Ambion, Austin, TX) at 42°C for 2h. Hybridization was carried out overnight in the same buffer containing probes prepared by nick-translation (Amersham Pharmacia Biotech Corp., Arlington Heights, IL) and using [α-^{32}P] dCTP. The DNA fragments used for probe preparation included: (a) the human SLPI RT-PCR product (399 bp); (b) the partial cDNA fragments for the cell cycle components cyclin D1 (482 bp), TGF-β1 (326 bp), Cdk4 (399 bp), PCNA (520 bp), p21 (283 bp) (40) generated by RT-PCR, using cDNAs from Ishikawa total RNA as template and primer sets that were synthesized based on the reported human cDNA sequences (GenBank); and (c) the *EcoR*I inserts of the plasmid DNAs obtained from TOPO cloning of differentially-expressed cDNAs identified by mRNA differential display (below). The membranes were sequentially washed twice in 2x saline sodium citrate (SSC), 0.1%SDS and in 0.1x SSC, 0.1%SDS at 42°C for 15 min each time. The blots were exposed to X-ray films using intensifying screens, and resultant hybridization signals were quantified using the Alpha Imager 2000 Documentation and Analysis System (Alpha Innotech Co., San Leandro, CA). The probe was removed after each hybridization reaction by washing the filter twice for 20 min each in 1% SDS solution heated at 100° C. A partial cDNA fragment (971 bp) corresponding to human GAPDH coding sequence (Tokunaga et al., 1987) was labeled by nick-translation and used to normalize for loading differences among samples.

mRNA Differential Display

Analysis of differentially expressed mRNAs among clonal sublines utilized the methodology of differential display-reverse transcriptase-polymerase chain reaction (ddRT-PCR) performed as previously described in publications from this laboratory

(Green et al., 1995; Min et al., 2002). DNA-free total RNAs (2 μg) isolated from the parental Ishikawa cell line and the clonal lines 13pIND, 11As, and 8As, were each subjected to reverse transcription using anchored 3' oligo (dT) primer sets (HIEROGLYPH™, GENOMYX Corp., Foster City, CA). One tenth of the reverse transcription reaction (20 μl) was used for PCR amplification in a reaction (20 μl total volume) containing 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT), 400 μM of each dNTP, 2.5 μCi of [γ-^{33}P] dATP, and two primers: 4 μM of one of T_{12} oligonucleotides and 4 μM of an arbitrary decamer, M13r-ARP1 (5'-CGACTCCAAG-3') or M13r-ARP2 (5'-GCTAGCATGG-3'). The PCR products from different primer combinations for each of the four cell lines examined were generated following previously described amplification conditions (Min et al., 2002), separated in non-denaturing polyacrylamide (4.5%) sequencing gels and visualized by autoradiography. A number of bands exhibiting differential expression were excised from the dried gels, and re-amplified by PCR using the appropriate primers. These fragments were used as probes in Northern blots, and were cloned into TOPO™ TA vector for nucleotide sequencing when established to be differentially expressed. The identities of the isolated clones were determined by homology comparisons with sequences in GenBank (www.ncbi.nlm.nih.gov/BLAST/).

Transient Transfection and Luciferase Assays

The full-length human SLPI coding region was released from the plasmid DNA pT_7T_3D-hSLPI (IMAGE: 796471; IncyteGenomics Inc., Palo Alto, CA) by digestion with *EcoR*I and *Not* I, and subsequently ligated into the pcDNA3 vector (Invitrogen) that was previously linearized with the same enzymes. The cyclin D1 reporter construct (–

1745CD1LUC in pcDNA3.1/Zeo$^+$ vector) contained the promoter and 5' regulatory sequences of the human cyclin D1 gene (Allan et al., 2001) and was generously provided by Drs. Richard G. Pestell and Chris Albanese (Albert Einstein College of Medicine, NY).

For transient transfections, the anti-sense subline 8As cells were seeded at a density of 6 x 10^5 cells/well in serum-containing medium. Twenty-four h thereafter, cells were incubated with reporter construct (-1745CD1LUC; 5 μg/well) and SLPI expression construct (pcDNA3-hSLPI; 0.5, 1 or 2μg/well) or corresponding empty vector (pcDNA3) in the presence of Hexadimethrine Bromide (polybrene, 5μl/well; Sigma Biochemicals), for 4-6 h in Hanks' Balanced Salt Solution (HBSS, pH 7.4). Cells were washed with HBSS once, treated with 25% dimethylsulfoxide (DMSO) in HBSS for 4 min to increase cell permeability, and then washed twice with the same buffer prior to their incubation in serum -containing MEM for an additional 48h. Luciferase activity (measured as relative light units, RLU) in cell lysates prepared using single-strength lysis buffer (Promega) was determined using the Promega Luciferase Assay system. Three independent transfection experiments were performed, with each experiment carried out in triplicate. Results were normalized to the protein content of each sample, and are presented as least-square means ± SEM. Protein concentration of extracts was determined by the Bradford method (Bradford 1976).

Parental Ishikawa cells were used in the co-transfection of porcine SLPI promoter-Luc reporter construct (containing 887 bp upstream promoter region, 5μg/well, Reed et al., 1996) and BTEB1 or BTEB2 mammalian expression construct, pcDNA3-BTEB1 (0.5μg/well, generously provided by Dr. Imataka, Kyoto University, Kyoto,

Japan) or pBK-CMV-BTEB2 (0.5μg/well, generously provided by Dr. Tina Teng, NIEHS/NIH, Research Triangle Park, N.C.), or their empty vector, pcDNA3 (0.5μg/well), pBK-CMV (0.5μg/well). Cells were seeded at 6 x 10^5 cells/well in serum-containing medium 24h before the transfection. The method of transient transfection and the luciferase assay were previously described in Chapter 4.

In studies where the incorporation of labeled thymidine was measured after transient transfection with the SLPI expression construct or empty vector, transfections of the clonal line 8As were carried out using Lipofectamine, following previously described protocols (Zhang et al., 2002).

Immunofluorescence and Western Blots

The clonal 13pIND and 8As lines were seeded on immunofluorescence chamber slides (Nalge Nunc International Corp., Naperville, IL) at a density of 1 x 10^5 cells. The following day, cells were washed with Phosphate-Buffered Saline (PBS), incubated with 4% paraformaldehyde for 10 min, rinsed with PBS three more times, and then incubated with 0.1% TritonX-100 in PBS for 10 min to increase nuclear permeability of antibody. Incubation with rabbit anti-human cyclin D1 polyclonal antibody (final concentration of 4 μg/ml; R&D Systems) or Normal Rabbit Serum (NRS) IgG (negative control; 4 μg/ml) diluted in 3% Bovine Serum Albumin (BSA)/PBS was carried out in a humidified box for 1h. Detection was performed with anti-rabbit FITC conjugated secondary antibodies (Sigma Biochemicals; 1:200 dilution) for 45 min. Cells were photographed using the Zeiss Axioplan 2 Fluorescence Microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY).

Standard Western blot techniques were used to determine the levels of PCNA in cell nuclear extracts prepared following previously described procedures (Gonzalez et al., 1994), using anti-PCNA monoclonal antibody (1:2000 dilution). SLPI protein in conditioned medium was detected using rabbit polyclonal anti-human SLPI antibody that was generously provided by Dr. P.S. Hiemstra (Leiden University, The Netherlands).

Ligand Blot Analysis

Confluent 13pIND and 8As clonal lines were incubated in serum-free medium, and conditioned medium was collected 24 h later. Conditioned medium was also collected from confluent 11As and 13pIND clonal lines incubated for 24, 48, and 72h in serum-free medium. The samples were concentrated, and aliquots corresponding to 30 μg of protein for each sample, were electrophoresed on 10% SDS-PAGE. Standard ligand blot procedures using ^{125}I IGF-I as probe (5 x 10^6 total counts per membrane) were followed (Lee et al., 1998).

Statistical Analysis

The intensities of hybridization bands obtained from Northern blots were normalized to those for the control probe GAPDH, and analyzed for differences using one-way ANOVA. The transfection data were normalized to cellular protein content, and analyzed by two-way ANOVA. Results are presented as least-squares means ± SEM. Differences with P values < 0.05 were considered statistically significant.

Results

Regulation of SLPI Gene Expression by BTEB1

A representative Northern blot of human SLPI transcripts, as well as corresponding GAPDH mRNA, in normal Hec-1-A cells, and in stablely transfected Hec-

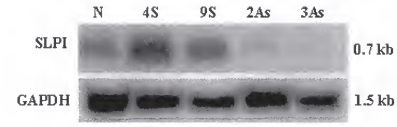
1-A sublines, namely the higher BTEB1 expressing lines (4S & 9S), and the lower BTEB1 expressing lines (2As & 3As), is shown in Figure 5-1 (panel A). When normalized to GAPDH hybridization signals (loading control), human SLPI mRNA levels were observed to be highest in 4S and 9As >normal (parental) Hec-1-A >2As and 3As (Figure 5-1, A). These findings indicate that SLPI gene expression is positively correlated with BTEB1 mRNA levels in Hec-1-A cells. Transient transfection studies using the porcine SLPI promoter (Reed et al., 1996) showed that BTEB1 directly increased porcine SLPI gene transcription (~2fold), whereas BTEB2 had no effect (Figure 5-1B).

Identification of SLPI Anti-sense Clonal Lines

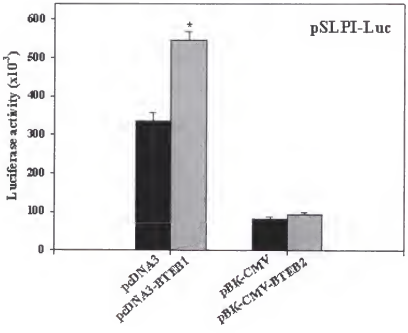
Clonal lines derived by stable transfection of Ishikawa cells with an expression construct containing the entire coding region of human SLPI in anti-sense orientation were confirmed for expression of this construct by RT-PCR using a primer set located within the pIND vector (forward) and the SLPI cDNA sequence (reverse), respectively (Figure 5-2A). The relative expression of the anti-sense construct for each clonal line was determined from ethidium bromide-stained agarose gels containing the generated 451 bp PCR fragment (Figure 5-2B) and was verified by Northern and Western blot analyses of corresponding cellular RNA and protein, respectively for SLPI (Figure 5-2C). The level of abundance of the 451 bp fragment in the Anti-sense (As) lines 8, 11, and 18 (8 > 11>18) was inversely correlated with their expression of endogenous SLPI mRNA and protein (8<11 <18). As expected, control lines (3pIND, 13pIND) stably expressing the empty vector had SLPI mRNA and protein levels that were indistinguishable from those of parental (untransfected) cells (Figure 5-2C). Since the functional consequence of

Figure 5-1. Regulation of SLPI gene expression by BTEB1. A, Northern blot analysis for SLPI mRNA abundance in normal (parental) Hec-1-A cells (N), sublines 4S and 9S, which are derived from normal Hec-1-A cells by overexpression of BTEB1, and sublines 2As and 3As, which are derived from normal Hec-1-A cells and have lower BTEB1 expression than parental cells. Parallel analysis for GAPDH mRNA was conducted to correct for equal RNA loading and integrity. B, Normal Ishikawa cells were seeded at $6x10^5$/well 24h before they were co-transfected with porcine SLPI promoter (-887)-Luc reporter construct (pSLPI-Luc) and the mammalian expression construct for BTEB1, BTEB2, or their corresponding empty vectors (pcDNA3 and pBK-CMV), respectively. Luciferase assay on cell lysates was carried out 48 h later. Three independent transfection experiments were carried out, with each experiment done in triplicate. Results were normalized to the protein content for each sample, and are presented as Least-square means ± SEM.

A


N
4S
9S
2As
3As
SLPI
0.7 kb
GAPDH
1.5 kb

B


pSLPI-Luc
Luciferase activity (x10³)
600
500
400
300
200
100
0
pcDNA3
pcDNA3-BTEB1
pBK-CMV
pBK-CMV-BTEB2

SLPI ablation was the major focus of the present study, the two lines that exhibited the most dramatic differences in SLPI gene expression, namely 8As and the control 13pIND, were selected for further analysis.

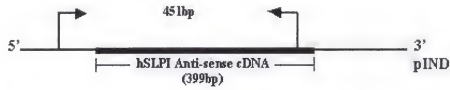
SLPI Functions as an Epithelial Cell Growth Factor

The effect of SLPI on subsequent epithelial cell number was evaluated for the two clonal lines that had undetectable (8As) and high (13pIND) levels of SLPI expression, respectively. Cells were seeded at the same density, transferred to serum-free medium to synchronize cell cycle stage, and then transferred back to serum-containing medium for the duration of the study. As shown in Figure 5-3A, cell growth rate was higher in the 13pIND line than in the 8As line at all the time points examined; indeed, 6 days after plating, the cell density observed for the control line was 6-fold higher on average than for the SLPI "knock-out" line. Consistent with this, cellular DNA synthesis, quantified by labeled thymidine incorporation was higher in 13pIND than 8As line (Figure 5-3B). The clonal line 11As shown earlier to express SLPI at levels between those of control and 8As lines (Figure 5-2C) exhibited DNA synthetic rates that were also mid-way between the two lines. These results indicated a positive correlation between levels of SLPI expression and epithelial cell proliferation.

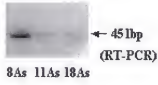
To further examine this relationship, the 8As clonal line that had undetectable expression of SLPI was transiently transfected with a human SLPI expression construct (pcDNA3-hSLPI) or empty vector (pcDNA3), and thymidine incorporation by transfected cells was measured. Cellular DNA synthesis in the 8As clonal line that was transfected with pcDNA3-hSLPI was greater than in cells transfected with empty vector

Figure 5-2. Expression of SLPI in stably transfected human Ishikawa clonal lines. A, Schematic representation of the entire coding region of human SLPI (hSLPI) (399 bp) inserted in the anti-sense orientation in pIND expression plasmid, and the location of the forward and reverse primers used in RT-PCR to confirm the expression of the chimeric construct. B, Analysis of chimeric anti-sense (As) SLPI mRNA expression by RT-PCR. cDNAs were synthesized from total RNA isolated from 8As, 11As, and 18As sublines, and used as templates in PCR reactions using primers described in the text. The expected PCR fragment of 451 bp is shown. C, Northern and Western blot analysis of SLPI in parental Ishikawa cells and clonal lines. Total RNA (30μg/lane) isolated from the indicated cell lines, was probed with either [α-^{32}P] hSLPI (399bp) or [α-^{32}P] hGAPDH (971 bp) cDNA fragments. The conditioned medium from the indicated cell lines after serum starvation for 24h was collected, concentrated and loaded (10μg/lane) onto 10% SDS-PAGE. Immunoreactive SLPI was detected using a rabbit anti-human SLPI polyclonal antibody (generously provided by Dr. P.S. Hiemstra, Leiden University Medical Center, The Netherlands) and the ECL detection system.

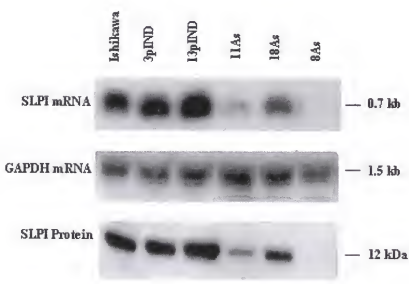
A


451bp
5'
3'
hSLPI Anti-sense cDNA
(399bp)
pIND

B


451bp
(RT-PCR)
8As 11As 18As

C

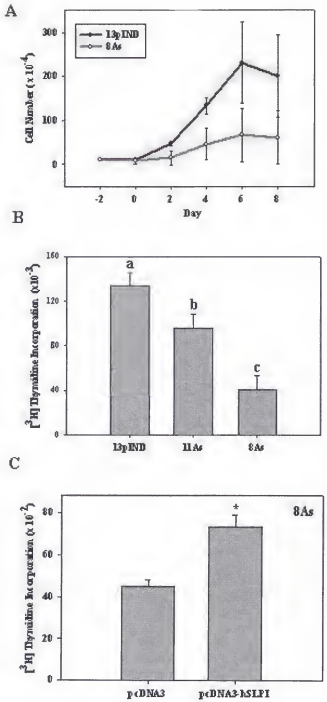

Ishikawa
3pIND
13pIND
11As
18As
8As
SLPI mRNA
0.7 kb
GAPDH mRNA
1.5 kb
SLPI Protein
12 kDa

(Figure 5-3C). These results indicate that SLPI directly promotes endometrial epithelial cell proliferation.

Expression Levels of Cyclin D1 and TGF-β1 are Dependent on SLPI

To determine if the modulation by SLPI of epithelial cell proliferation involves specific changes in cell cycle- and growth- associated gene expression, the mRNA levels for a number of cell cycle-related proteins were quantified in several clonal As-SLPI lines and for comparison, in the parental Ishikawa cell line and the control pIND sublines. As shown in Figure 5-4A, the levels of cyclin D1 and of TGF-β1, mRNAs were positively and negatively correlated, respectively with those of SLPI. When normalized to the expression of the control GAPDH gene, which was unaffected by cellular SLPI expression, these positive (cyclin D1) and inverse (TGF-β1) relationships with SLPI were most evident (Figure 5-4B). In particular, the 8As clonal line, which had undetectable SLPI gene expression, had the lowest and highest levels, respectively of cyclin D1 and TGF-β1 mRNAs. By contrast, the expression of Cdk4, PCNA, and p21 genes were relatively unchanged in all clonal lines, irrespective of SLPI gene expression levels. Immunofluorescence with a specific antibody against human cyclin D1 was used to evaluate if distinct differences exist in the levels of this protein in 8As and control (13pIND) sublines. Consistent with the differences noted in cyclin D1 mRNA levels, the nuclear abundance of cyclin D1 in 8As was less than that observed for 13pIND (Figure 5-5). In particular, almost all 13pIND cells exhibited staining while those for 8As were more irregular and spotty. The specificity of the antigen-antibody reaction was verified by virtue of the undetectable nuclear staining observed in both lines when control rabbit IgG was used in place of the test antibody. Further, the inductive effect of SLPI was

Figure 5-3. Distinct growth parameters of the Ishikawa sublines are dependent on cellular SLPI content. A. Control (13pIND) and anti-sense SLPI (8As) sublines were seeded at a density of $1.2x10^5$ cells/well in serum-containing medium (-2 Day). Cells were allowed to adhere to the plates for 24h, and medium was changed to serum-free. After an additional 24h, cells received fresh medium containing 10% FBS (0 Day). Every other day for a total of 8 days, the cells were counted under a microscope using a hemocytometer. B. DNA synthesis in control (13pIND) and anti-sense SLPI (11As, 8As) sublines. Cells were seeded at the same density ($6x10^5$/well) and were allowed to attach to the dishes. After serum-starvation for 24h, cells were changed to serum-containing medium for another 24h. Cells were pulse-labeled with [^{3}H] thymidine for 4 h and the assay for incorporation of label was carried out as described in the text. C. DNA synthesis in the 8As subline in the presence or absence of human SLPI expression construct. Cells were transiently transfected with either the SLPI expression construct (pcDNA3-hSLPI) or empty vector (pcDNA3) and assay for [^{3}H] thymidine incorporation was carried out 24 h thereafter. Data (mean ± SEM) were from three independent experiments, where each experiment was carried out in triplicate wells.


A
13pEND
8As
Cell Number (x 10^-4)
300
200
100
0
-2
0
2
4
6
8
Day
B
[3H] Thymidine Incorporation (x10^-3)
160
120
80
40
0
a
b
c
13pEND
11As
8As
C
[3H] Thymidine Incorporation (x10^-2)
80
60
40
20
0
*
8As
pcDNA3
pcDNA3-hSLPI

selective for cyclin D1 since the protein levels of PCNA were not different in nuclear extracts prepared from 8As and 13pIND sublines in agreement with the PCNA mRNA data (Figure 5-4, 5-6).

SLPI Modulates Expression of Growth-associated Genes

To further explore the mechanism behind SLPI regulation of epithelial cell proliferation, we used the methodology of mRNA differential display to isolate differentially expressed growth-related genes. We identified an mRNA (A-16 transcript) whose expression was lower in cells expressing more SLPI. This mRNA is 4.5 kb in length (Figure 5-7), and after subcloning (TOPO vector) and DNA sequencing (Figure 5-8), it was identified by homology comparisons with sequences in GenBank (www.ncbi.nlm.nih.gov/BLAST/) to encode a partial 3'UTR of lysyl oxidase (LOX) (Figure 5-9), a key enzyme involved in the control of collagen and elastin maturation (Kagan and Trackman, 1991), and which has been shown recently to act as a tumor suppressor by virtue of its ability to inhibit the oncogene p21 (ras) gene expression and pathways (Kenyon et al., 1991; Ekholm et al., 2000; Chinoy et al., 2000) (Fig 5A). Another mRNA (A-10 transcript, 1.3 kb in length) whose expression was lower in the 8As control line was also isolated. However, the identity of this mRNA is presently unknown since its sequence does not correspond to any of those currently deposited in GenBank (Figure 5-7, 5-10).

We also employed the IGF ligand blot procedure to evaluate if changes in the levels of IGF-binding proteins can be correlated with cellular SLPI, since Ishikawa cells are autocrine targets of IGF-I (Reynolds et al., 1998). The anti-sense SLPI lines (8As and 11As) and the control line 13pIND demonstrated the presence of two major IGF

binding proteins, namely IGFBP-2 and IGFBP-3 in the conditioned medium. However, while the levels of IGFBP-2 did not change among the three sublines, those for IGFBP-3 were increased in the anti-sense lines (Figure 5-11). Moreover, the levels of IGFBP-3 appeared to be inversely correlated with those of SLPI, since 11As (higher SLPI gene expression) had less secreted IGFBP-3 than did 8As.

To examine whether addition of SLPI to the medium of anti-sense lines can alter their patterns of gene expression to mimic that of the control (pIND) line, changes in gene expression in sublines to which were added recombinant human (rh) SLPI twice over a 48-h interval were measured by Northern blots. Consistent with earlier observations (above), the basal expression levels of the TGF-β1, IGFBP-3 and LOX genes were negatively correlated with endogenous SLPI expression, while that of cyclin D1 was positively correlated (Figure 5-12). Addition of rhSLPI had an effect only on cyclin D1 mRNA levels, which were increased by ~ 3-fold relative to basal levels, but only in the 8As line. By contrast, the expression of the other genes that were similarly evaluated in all sublines were unaffected by rhSLPI addition.

To assess if the effects of SLPI on cyclin D1 gene expression occurred through a signaling pathway mediated by a plasma membrane-activated second messenger subsequent to SLPI binding, the consequence of increasing intracellular levels of SLPI on the expression of cyclin D1 as well as other genes was examined by transient transfection of a human SLPI expression vector into the 8As line. As shown in Figure 5-13, the expression vector significantly increased, albeit modestly, the levels of cyclin D1, but not of TGF-β1, IGFBP-3 and LOX, mRNAs relative to empty vector in this subline. Interestingly, basal SLPI mRNA levels were increased by at least 4-fold with transfected

SLPI expression vector relative to empty vector. Together, these results suggest that SLPI has a direct regulatory role on cyclin D1 gene expression, while its effects on the expression of the other genes may be mediated by indirect mechanisms.

SLPI Affects Cyclin D1 Promoter Activity

The mechanism behind SLPI regulation of cyclin D1 gene expression was examined by evaluating whether SLPI modulates cyclin D1 promoter activity. The anti-sense line 8As was transiently co-transfected with the cyclinD1-Luc reporter construct and human SLPI expression vector or corresponding empty vector, and luciferase activity was measured in resultant cell lysates. SLPI had a dose-dependent effect on cyclin D1 promoter activity, with no effect of the added expression vector at 0.5 μg, a modest inductive effect at 1.0 μg, and a dramatic inhibitory effect at 2 μg (Figure 5-14). This suggests that SLPI regulates cyclin D1 gene expression at the level of transcription, although the direction of regulation (transactivation or transrepression) appeared to be dose-dependent.

TGF-β1 Opposes SLPI Effects on Gene Expression

The lack of a direct effect of SLPI, whether added extracellularly or produced intracellularly, on the expression of IGFBP-3 and LOX genes, despite the observed negative correlation of their expression with that of SLPI in anti-sense lines suggest indirect mode(s) of SLPI control. Since the expression levels of SLPI and TGF-β1 genes are inversely correlated in the Ishikawa sublines described here, we examined whether TGF-β1 had a direct effect on IGFBP-3, LOX, and cyclin D1 gene expression, respectively. In these studies, parental Ishikawa cells were incubated in serum-free medium with or without added rhTGF-β1 for 24 h, and total cellular RNA from treated

and control cells were analyzed for expression of these genes (Figure 5-15) by Northern blotting. Relative to untreated cells, TGF-β1 decreased SLPI (~1.5-fold) and IGFBP-3 (~2.5-fold), increased LOX (~3-fold) and its own (~2.5-fold), and had no effect on cyclin D1, mRNA levels (Figure 5-16). These findings suggest a regulatory loop between SLPI and TGF-β1,whereby induction of TGF-β1 gene expression is a direct or an indirect consequence of diminished SLPI expression, and results in increased levels of LOX.

Discussion

Previous study demonstrated that increased expression levels of BTEB1 in the endocarcinoma cell line Hec-1-A were correlated with increased proliferative status. The increased proliferation seen in high BTEB1-expressing cells compared to those with low BTEB1 expression, was associated with increased traversal of the cell cycle, consistent with these cells' enhanced expression of cyclin D1 and other growth-associated proteins (Zhang et al., 2001; Simmen et al., 2002). Thus, BTEB1 can function to modulate cell growth, and present study outlines a possible mechanism by which BTEB1 modulates cell proliferation, in part, through regulation of SLPI gene expression. In this study, SLPI gene expression as measured at the level of mRNA and promoter activity was found to increase with higher levels of BTEB1. Compared to BTEB1, BTEB2 had no effect on SLPI promoter activity. Taken together, these results suggest an important role for BTEB1 in the regulation of uterine endometrial epithelial cell proliferation.

This study provides a novel mechanism by which secretory leukocyte protease inhibitor (SLPI), a multi-substrate anti-protease member of the serpin family, functions as an epithelial cell growth factor. Several lines of evidence presented herein suggest that

Figure 5-4. Expression of cell cycle-associated genes in parental Ishikawa and derived clonal lines with distinct SLPI expression. Cells were grown to confluence in serum-containing medium, and then collected for RNA isolation. A, Total RNA (30 μg/lane) were analyzed for message content of the indicated genes for each subline (panel A) using [α-^{32}P] labeled cDNA fragments as probes. B, The bar graphs show the levels of the individual transcripts after normalization of the band intensities with that of GAPDH.

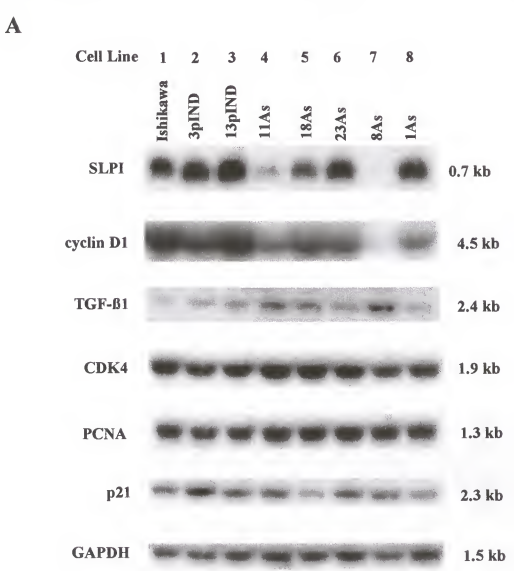
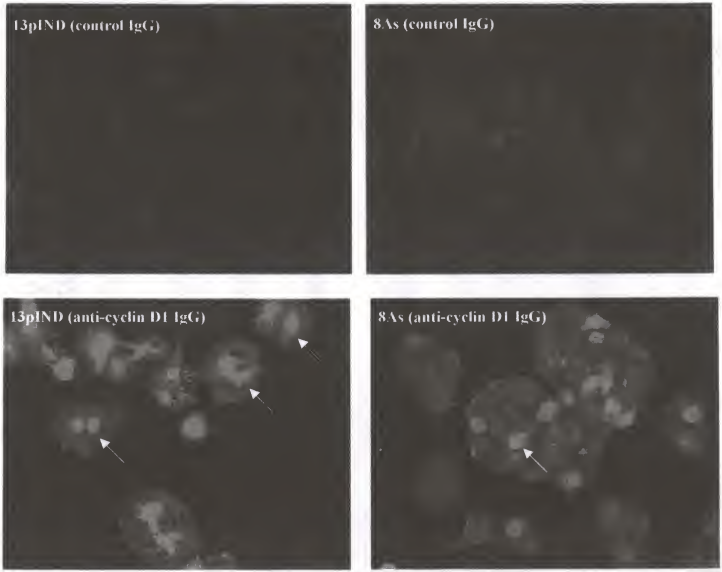

A
Cell Line 1 2 3 4 5 6 7 8
Ishikawa
3pIND
13pIND
11As
18As
23As
8As
1As
SLPI
0.7 kb
cyclin D1
4.5 kb
TGF-ß1
2.4 kb
CDK4
1.9 kb
PCNA
1.3 kb
p21
2.3 kb
GAPDH
1.5 kb
B
Relative Intensity
SLPI
cyclin D1
TGF-ß1
CDK4
PCNA
p21
Cell line 1 2 3 4 5 6 7 8

Figure 5-5. Abundance of cyclin D1 proteins in control (13pIND) and SLPI-deficient (8As) sublines. Cells were seeded at a density of $1x10^5$/well on immunofluorescence chamber slides. The following day, cells were incubated with rabbit anti-human cyclin D1 polyclonal IgG (4μg/ml) (bottom panels) or Normal Rabbit Serum IgG (4μg/ml) (top panels), followed by FITC-conjugated secondary antibodies. Photos were taken using the Zeiss Axioplan 2 Fluorescence Microscope.


13pIND (control IgG)
8As (control IgG)
13pIND (anti-cyclin D1 IgG)
8As (anti-cyclin D1 IgG)

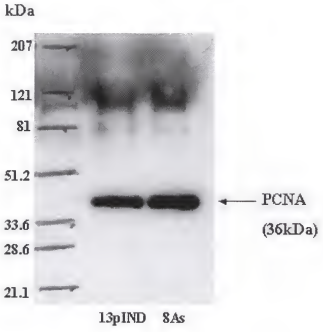


Figure 5-6. Abundance of PCNA proteins in control (13pIND) and SLPI-deficient (8As) sublines. Nuclear extracts prepared from 13pIND and 8As cells (100 μg/lane) were analyzed on Western blots using PCNA monoclonal antibody (1:2000 dilution). The PCNA protein of M_r 36 kDa is shown.

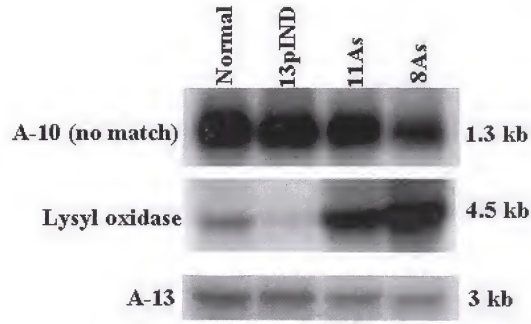


Figure 5-7. Differential expression of growth-associated genes in stably transfected Ishikawa sublines. Total cellular RNA isolated from parental Ishikawa cells and clonal lines stably transfected with empty vector (13pIND) or anti-sense SLPI expression construct (11As, 8As), were subjected to mRNA differential display RT-PCR as described under "Materials and Methods". DNA fragments representing three potential differentially expressed transcripts were used as probes in Northern blots. Nucleotide sequence analysis identified the 4.5 kb transcript as that for lysyl oxidase (LOX). The ddRT-PCR product derived from the 3'UTR of exon 7 of this gene. The identity of the 1.3 kb transcript for clone A-10 is currently unknown. Clone A-13 (3 kb) is a false positive from ddRT-PCR.

DD-RT-PCR clone A-16 sequence

CAAT TTCACACAGG AGCTAGCATG GTTAGAGGGG CTAGAGATGG
ACAGTCATCA ACTGGCGGAT ATAGCGGTAC ATATGATCCT TAGCCACCAG
GGCACAAGCT TACCAGTAGA CAATACAGAC AGAGCTTTTG TTGAGCTGTA
ACTGAGCTAT GGAATAGCTT CTTTGATGTA CCTCTTTGCC TTAAATTGCT
TTTTAGTTCT AAGATTGTAG AATGATCCTT TCAAATTGTA ATCTTTTCTA
ACAGAGATAT TTTAATATAC TTGCTTTCTT AAAAAACAAA AAAACTACTG
TCAGTATTAA TACTGAGCCA GACTGGCATC TACAGATTTC AGATCTATCA
TTTTATTGAT TCTTAAGCTT GTATTAAAAA CTAGGCAATA TCATCATGGA
TACATAGGAG AAGACACATT TACAATCATT CATTGGGCCT TTTATCTGTC
TATCCATCCA TCATCATTTG AAGGCCTAAT ATATGCCAAG TACTCACATG
GTATGCATTG AGACATAAAA AAGACTGTCT ATAACCTCAA TAAGTATTAA
AAATCCCATT ATTACCCATA AGGTTCATCT TATTTCATTT TTAGGGAATA
AAATTACATG TCTATGAAAT TTCAATTTTA AGCACTATTG TTTTTCATGA
CCATAATTTA TTTTTAAAAA TAAATTAAAG GTTAATTATA TGCATGTATG
TATTTCTAAT AATTAAAAAT GTGTTCAATC CCTAAAAAAA AAAA

Figure 5-8. A-16 transcripts sequence. A-16 cDNA fragment was identified to have a different expression level between Normal, 13pIND, 11As and 8As Ishikawa cell lines and sublines using DD-RT-PCR method. The cDNA fragment were then cloned into pCRII-TOPOTM vector for nucleotide sequencing.

gi|733134|gb|U22384.1|HSU22384 Homo sapiens lysyl oxidase gene, partial cds and 3' UTR (Length = 3585)

Score = 795 bits (401), Expect = 0.0
Identities = 434/440 (98%), Gaps = 5/440 (1%)
Strand = Plus / Plus

```
Query:335   ctactgtcagtattaatactgagccagactggcatctacagatttcagatctatcatttt 394
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Sbjct:1796  ctactgtcagtattaatactgagccagactggcatctacagatttcagatctatcattt- 1854

Query: 395  attgattcttaagcttgtattaaaaactaggcaatatcatcatggatacataggagaaga 454
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1855 attgattcttaagcttgtattaaaaactaggcaatatcatcatggatacataggagaaga 1914

Query: 455  cacatttacaatcattcattggg-ccttttatctgtctatccatccatcatcatttgaag 513
            ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
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Query: 693  ataatttatttttaaaaataaattaaaggttaattatatgcatgtatgtatttctaataa 752
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2153 ataatttatttttaaaaataaattaaaggttaattatatgcatgtatgtatttctaataa 2212

Query: 753  ttaaaaatgtgttcaatccc 772
            ||||||||||||||||||||
Sbjct: 2213 ttaaaaatgtgttcaatccc 2232
```

Figure 5-9. The sequence comparison between A-16 clone and human lysyl oxidase gene partial cds and 3'UTR. The A-16 cDNA fragment isolated from DD-RT-PCR was subcloned into pCRII-TOPO ^{TM} cloning vector and sequenced. The identity of this clone was determined by homology comparison with sequences reported in GenBank (www.ncbi.nlm.nih.gov/BLAST/). The upper line is the A-16 cDNA fragment sequence, the lower line is the partial 3'UTR of lysyl oxidase gene sequence.

DD-RT-PCR clone A-10 sequence

```
TGGGCCCTCT AGATGCATGC TCGAGCGGCC GCCAGTGTGA TGGATATCTG
CAGAATTCGC CCTTGACCAC TATAGGGCTT TTTTTTTTTT CGCAAATAGC
ACTTTTTATT TGCCACTATT TGAAGTCTGA ACTTTAAACA GATTCTTGGA
CTGGTGGTTC ATATCCATCA GCTCGTTCAA CTTTAGCACC TGTCTCGTCC
CCAGTGGCTT TTCCAGAACT ACTGCCTTCA CCATGCTAGC TCCTGTGTGA
AATTGTAAGG GCGAATTCCA GCACACTGGC GGCCGTTACT AGTGGATCCG
AGCTCGGTAC CAAGCTTGAT GCATAGC
```

Figure 5-10. A-10 transcript sequence. A-10 cDNA fragment was identified to have a different expression level between Normal, 13pIND, 11As and 8As Ishikawa cell lines and sublines using the DD-RT-PCR method. The cDNA fragment was cloned into pCRII-TOPOTM vector for nucleotide sequencing.

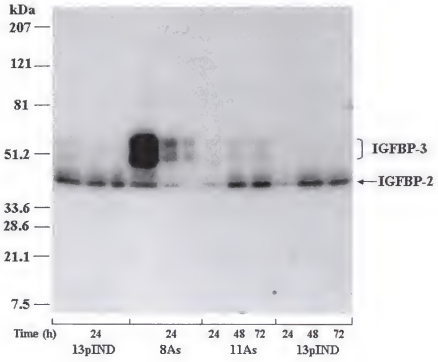
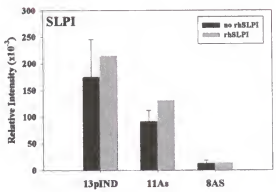
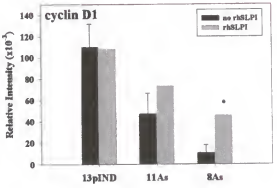


Figure 5-11. Different abundance of IGFBP-3 protein in stably transfected Ishikawa sublines. Confluent 13pIND and 8As clonal lines were incubated in serum-free medium, and conditioned medium was collected 24h later. Conditioned medium was also collected from 11As and 13pIND clonal lines incubated for 24, 48, and 72h in serum-free medium. The samples (30μg protein/lane) were concentrated, and subsequently analyzed for presence of secreted IGF-binding proteins using [^{125}I]-IGF-1 as a probe in ligand blots. The two radioactive bands of ~ M_r 51 kDa represent glycosylation variants of IGFBP-3 protein, while the lower band of M_r 36 kDa is IGFBP-2.

Figure 5-12. Effects of exogenously added rhSLPI on gene expression by control and SLPI-deficient clonal lines. Control (pIND) and anti-sense SLPI (11As, 8As) cell lines were grown to confluence, and then transferred to serum-free medium. After 24h, cells received either human SLPI (100ng/ml) or buffer alone twice at 24h intervals. Cells were collected 24h after the last treatment. Total RNA were isolated from these cells, and analyzed on Northern blots using [α-^{32}P] labeled inserts for SLPI, cyclin D1, TGF-β1, IGFBP-3, and LOX cDNAs, as probes. The bar graphs (LSM$\pm$ SEM) represent the hybridization intensities of each transcript after normalizing to that of GAPDH.


SLPI
no rhSLPI
rhSLPI
Relative Intensity (x10⁻³)
300
250
200
150
100
50
0
13pIND
11As
8AS


cyclin D1
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rhSLPI
Relative Intensity (x10⁻³)
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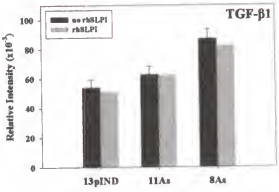
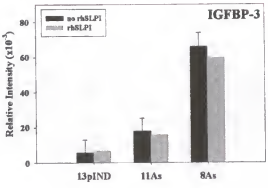
120
100
80
60
40
20
0
13pIND
11As
8As


TGF-β1
no rhSLPI
rhSLPI
Relative Intensity (x10⁻³)
100
80
60
40
20
0
13pIND
11As
8As


IGFBP-3
no rhSLPI
rhSLPI
Relative Intensity (x10⁻³)
80
60
40
20
0
13pIND
11As
8As

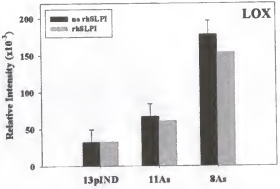
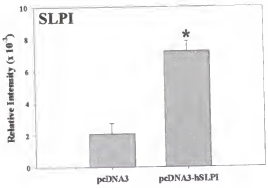
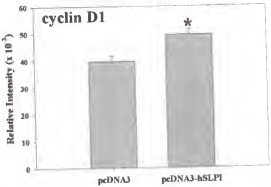
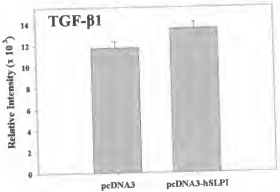
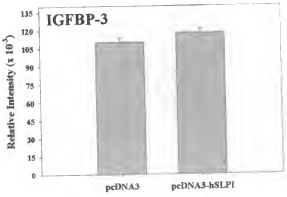

LOX
no rhSLPI
rhSLPI
Relative Intensity (x10⁻³)
200
150
100
50
0
13pIND
11As
8As

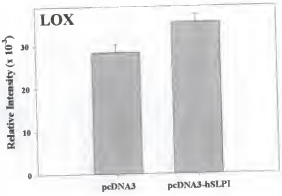
Figure 5-13. Effects of endogenous SLPI on gene expression by SLPI-deficient clonal lines. Cells at 70% confluence were transiently transfected with either empty vector (pcDNA3) or hSLPI expression construct (pcDNA3-hSLPI) using Lipofectamine, following procedures described in the text. Northern blot analysis was carried on total RNAs isolated from these cells, and analyzed as described above (Figure 5-12). Each experiment was repeated three times.


SLPI
Relative Intensity (x 10³)
10
8
6
4
2
0
*
pcDNA3
pcDNA3-hSLPI


cyclin D1
Relative Intensity (x 10³)
60
50
40
30
20
10
0
*
pcDNA3
pcDNA3-hSLPI


TGF-β1
Relative Intensity (x 10³)
14
12
10
8
6
4
2
0
pcDNA3
pcDNA3-hSLPI


IGFBP-3
Relative Intensity (x 10³)
135
120
105
90
75
60
45
30
15
0
pcDNA3
pcDNA3-hSLPI


LOX
Relative Intensity (x 10³)
30
20
10
0
pcDNA3
pcDNA3-hSLPI

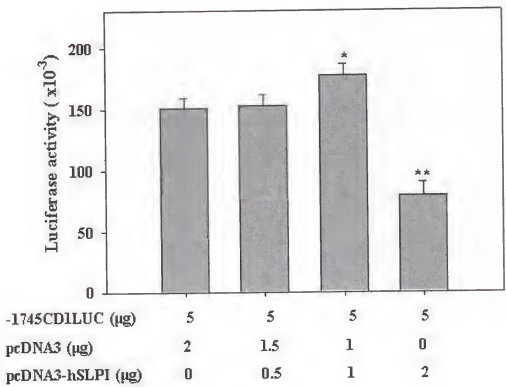


Figure 5-14. Effect of SLPI on cyclin D1 promoter activity. The 8As subline was seeded at a density of $6x10^5$ cells/well. Twenty-four hours after, cells were transiently co-transfected with –1745CD1LUC reporter construct (5µg/well) and SLPI expression construct (pcDNA3-hSLPI; 0.5, 1 or 2 µg/well) or corresponding empty vector pcDNA3, and luciferase assay on cell lysates was carried 48 h later. Three independent transfection experiments were carried out, with each experiment done in triplicate. Results were normalized to the protein content for each sample, and are presented as Least-square means ± SEM.

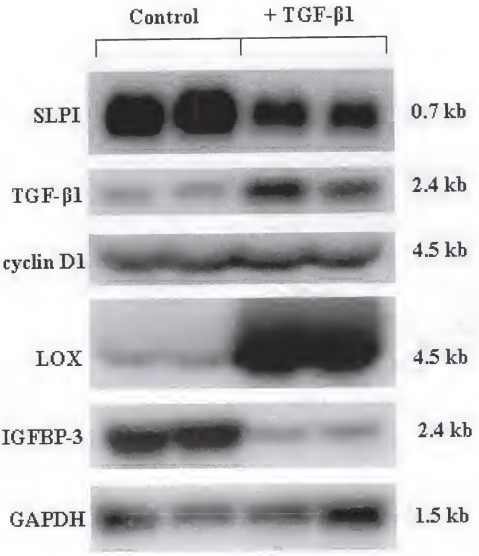
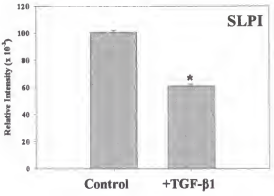
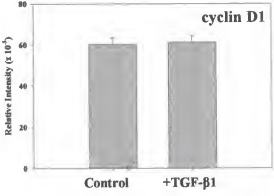
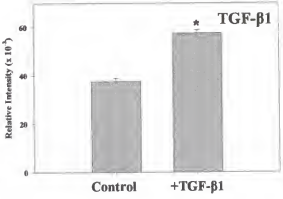


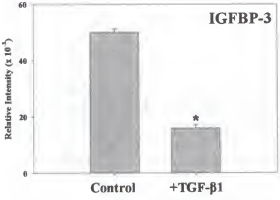
Figure 5-15. Effects of TGF-β1 on Ishikawa cell gene expression. Parental Ishikawa cells grown in serum-containing medium until 90% confluence were transferred to serum-free medium for 24h, prior to exogenous treatment with human TGF-β1 (10 ng/ml). Twenty-four h later, cells were collected, and total RNA isolated from these cells were analyzed by Northern blots for abundance of SLPI, TGF-β1, cyclin D1, LOX, IGFBP-3, and GAPDH transcripts. The Northern blot hybridization signals are shown in this figure.

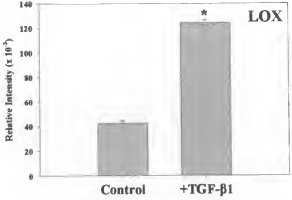
Figure 5-16. Effects of TGF-β1 on Ishikawa cell gene expression. This figure is the quantitative summary of the densitometric data from Figure 5-15, and the bar graphs (LSM± SEM) represent the hybridization intensities of each transcript after normalizing to that of GAPDH.


SLPI
Relative Intensity (x 10⁻³)
120
100
80
60
40
20
0
*
Control
+TGF-β1


cyclin D1
Relative Intensity (x 10⁻³)
80
60
40
20
0
Control
+TGF-β1


TGF-β1
Relative Intensity (x 10⁻³)
60
40
20
0
*
Control
+TGF-β1


IGFBP-3
Relative Intensity (x 10⁻³)
60
40
20
0
*
Control
+TGF-β1


LOX
Relative Intensity (x 10⁻³)
140
120
100
80
60
40
20
0
*
Control
+TGF-β1

the growth-regulatory role of SLPI, as measured by its ability to increase cell number as well as DNA synthesis, is mediated by its activation as well as its inhibition of distinct cell cycle and growth-associated gene expression. In particular, our findings that: 1) SLPI increased cyclin D1 gene expression at the level of this gene's promoter activity; 2) cellular SLPI levels are inversely correlated with those of TGF-β1 mRNA and IGFBP-3 mRNA and secreted protein; 3) the expression of the gene encoding lysyl oxidase (LOX), an inhibitor of the proto-oncogene ras gene expression (Ekholm et al., 2000; Chinoy et al., 2000) is markedly up-regulated in sublines with undetectable SLPI; and 4) TGF-β1 inhibits SLPI gene expression, are consistent with a regulatory role for SLPI in growth-signaling pathways. The diverse actions of SLPI highlighted here expand the repertoire of SLPI-regulated genes and encoded products, which previously have included the nuclear factor (NF)-κB inhibitor IκBβ (Lentsch et al., 1999) and prostaglandin H2 synthase-2 (Zhang et al., 1997), to those linked to growth control, and implicate distinct mechanisms employed by SLPI in modulating cell function and phenotype.

Perhaps a most intriguing finding is the identification of cyclin D1 as a downstream target of SLPI. The ability of SLPI to induce cyclin D1 expression occurred at the levels of its mRNA, protein, and promoter activity; is highly specific as evident from the lack of comparable effects on the expression levels of other G1 phase-associated genes such as Cdk4, PCNA, and p21; and was observed irrespective of the mode of SLPI delivery (intracellular or extracellular). A supportive role for SLPI in growth processes had been previously observed in epithelial and fibroblastic cell types (Kikuchi et al., 2000; Badinga et al., 1999), albeit the molecular mechanism underlying this function remained unclear. Members of the tissue inhibitor of metalloproteinase (TIMP) family,

specifically TIMP-1 and TIMP-2, exhibit growth-promoting activities, which appear to be mediated by signal transduction pathways independent of their protease inhibitor functions and involve cAMP (Hayakawa et al., 1992; Hayakawa et al., 1994; Guedez et al., 2001). The immediate and direct route for SLPI to modulate cell cycle progression via regulation of cyclin D1 expression is consistent with a mechanistically distinct pathway from that utilized for its protease inhibitory function, albeit this contrasts with previous findings of the requirement for this protein's trypsin inhibitory activity in the up-regulation of hepatocyte growth factor gene expression (Kikuchi et al., 2000). Interestingly, SLPI transactivation of the cyclin D1 gene appears to be dose-dependent, and was markedly reversed by a 2-fold increase in the amount of transfected SLPI vector. Although the reason for this is presently unclear, this may be related in part, to a previously reported activity of SLPI to inhibit the nuclear accumulation of NF-κB via its maintenance of the expression of IκBβ, a crucial step in NF-κB activation (Lentsch et al., 1999). Since NF-κB is a transactivator of cyclin D1 gene expression (Guttridge et al., 1999; Hinz et al., 1999; Joyce et al., 1999), a reduction in its nuclear levels secondary to its sequestration in the cytoplasm by SLPI-enhanced expression of IκBβ, might underlie the opposing consequence of SLPI on cyclin D1 promoter activity. Nevertheless, it is worthwhile to note that the changes in cyclin D1 transcriptional activity with SLPI are relatively modest. This finding coupled with the observation that the 8As subline with lower or undetectable SLPI gene expression are competent to grow, albeit at a much diminished rate than cells with robust expression of this anti-protease, is indicative of a contributory rather than a critical role for SLPI, in growth regulation. Another potential (and novel) mechanism behind SLPI control of cell growth as demonstrated here, may

involve its effective inhibition of the expression of a number of growth-suppressor genes. The latter include: 1) TGF-β1, a potent inhibitor of epithelial cell proliferation; 2) LOX, also known as ras-recision gene (Kenyon et al., 1991), which down-regulates the expression of the proto-oncogene ras, a component of the mitogen-activated protein kinase signaling pathway (Moodie et al., 1993; Itoh et al., 1993); and 3) IGFBP-3, a major factor in the control of IGF-I bio-availability and hence, of its growth promoting activity and a pro-apoptotic factor in its own right (Rechler and Clemmons, 1998). In this regard, we observed an inverse correlation between the expression of these genes and that of SLPI, at the levels of mRNA (TGF β1, LOX, IGFBP-3) or protein (IGFBP-3), although attempts to establish a causal relationship by the addition of rhSLPI to 8As cells in culture or by transient transfection of human SLPI expression construct to these cells, did not yield the anticipated effects. Since these same treatments resulted in the induction of cyclin D1 gene expression, these findings suggest that the regulatory mechanisms underlying the control of these genes' expression by SLPI, unlike that of cyclin D1, occur indirectly or via more long-term changes in cell phenotype. For LOX, this is a likely possibility since it is a known transcriptional target of TGF-β1 (Gacheru et al., 1997). The intermediate steps leading to the inhibition of TGF-β1 and IGFBP-3 gene expression upon increased expression of SLPI cannot be deduced from the present studies, although a regulatory loop arising from TGF-β1 inhibition of SLPI gene expression as demonstrated here, is consistent with reports observed for a bronchial epithelial cell line (Jaumann et al., 2000). In further agreement with the present results, a negative correlation between SLPI and TGF-β1 was reported in SLPI null mice (Ashcroft et al., 2000). In those studies, however, the consequence of the loss of SLPI expression was

observed at the level of TGF-β activation, rather than at the level of mRNA induction as reported here. Although the concentration of bioactive TGF-β1 was not measured in the present study, the parallel rise in protein (*in vivo*) and transcript (*in vitro*) levels of this growth inhibitor after ablation of the SLPI gene is clearly indicative of the physiological relevance of the SLPI/TGF-β linkage.

Based on the observations from this study, we propose a model that illustrates the regulation by SLPI of cell proliferation via its positive and negative influence on the expression of genes with known growth-regulatory activities (Fig 5-17). In this model, BTEB1 activates cell proliferation through increasing SLPI gene expression, and consequently, cyclin D1 gene expression increased by the direct effect of SLPI. SLPI inhibits the expression of the growth suppressors IGFBP3 and TGF-β1 genes, although the mechanism is likely indirect and may entail the participation of yet unknown intermediary steps. Further, SLPI negatively regulates LOX gene expression through a TGF-β1 mediated pathway. The end-result of this multiple regulation is the synergistic induction of cell proliferation. Thus, despite the modest effect of SLPI on the expression of individual genes, the sum total of the collective SLPI actions is the generation of a growth stimulus.

Several important questions are raised by the studies described here. One relates to the signaling pathway by which SLPI elicits its proliferative effects. Our finding that the addition of SLPI to culture medium resulted in the up-regulation of cyclin D1 gene expression is indicative of an integral membrane-situated "SLPI" receptor/binding protein, which may or may not be functionally-related to the recently described SLPI binding protein identified as scramblase (Zhou et al., 1997; Zhao et al., 1998; Tseng and

Tseng, 2000). Also consistent with the latter is the ability of transiently transfected SLPI expression construct that includes the signal sequence for SLPI secretion, to similarly enhance cyclin D1 gene expression. It has been noted that SLPI's three-dimensional structure characterized by four disulfide bonds is suggestive of its ability to utilize membrane receptor (s) to mediate its biological actions (Jin et al., 1997), however, the nature and identity of this membrane component is presently unknown. Further, the similar inhibitory effects of TGF-β1 and SLPI on IGFBP-3 expression are not entirely consistent with their opposing consequences on cell proliferation. However, this apparent discrepancy might be related to the nature of the parental cell line (Ishikawa) utilized in this study. In a number of adenocarcinoma cell lines, abnormal TGF-β response could be generated in a gene-specific context (Park et al., 2000; de Andrade et al., 2001; Breier et al., 2002). Finally, it would be of interest to ascertain whether other growth-regulatory genes mediate the proliferative effects of SLPI, and if their encoded products delineate sensitivity to SLPI in cell types other than the endometrial epithelial cells used here. Future studies in our laboratories utilizing the microarray approach will address these questions.

In summary, the generation and subsequent use of an SLPI "knock-out" *in vitro* model using the Ishikawa cell line, has allowed the elucidation of the molecular mechanisms behind the proliferative function ascribed to SLPI. Our data suggest that this newly described pathway involves the up- and down-regulation by SLPI, respectively, of the expression of positive (cyclin D1) and negative (TGF-β1, LOX, IGFBP-3) growth-associated factors via direct and indirect mechanisms. Our findings may have physiological relevance to further understanding the growth control of normal epithelium

as well as the lack thereof in certain epithelial-derived carcinomas wherein high expression of SLPI are manifest.

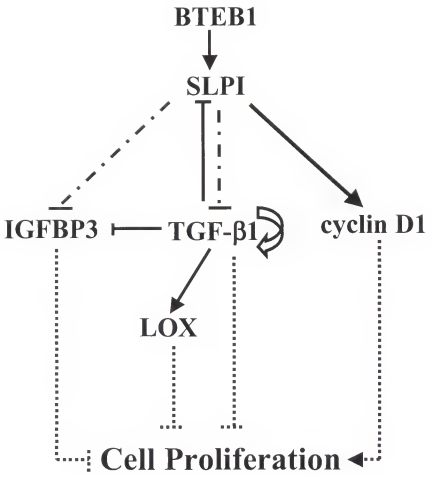


Figure 5-17. Model for the regulation of endometrial epithelial cell proliferation by BTEB1 through the SLPI pathway. The model illustrates that BTEB1 increases SLPI expression, and then, SLPI induces the expression of cylin D1 and inhibits that of IGFBP-3, TGF-β1 and LOX genes. The designations are as follows: lines with arrow, positive regulation; blocked lines, negative regulation; solid lines, direct regulation; broken lines, indirect regulation; dotted lines, previously published data; other lines; data from the present study. The arrow around TGF-β1 indicates auto-regulation.

CHAPTER 6
SUMMARY AND CONCLUSIONS

The purpose of the studies described in this dissertation was to further clarify the role of BTEB1 in the regulation of gene expression and the consequent effects on uterine endometrial epithelial cell differentiation and proliferation during pregnancy. These studies investigated: a) the regulation of BTEB1 gene expression by pregnancy hormones, estrogen (E) and progesterone (P); b) the contribution of BTEB1 to progesterone-responsive gene transcription via its interaction with the progesterone receptor isoform (PR)-B; and c) mechanism(s) underlying the modulation of uterine endometrial epithelial cell proliferation by SLPI, a pregnancy-associated, BTEB1 downstream target gene product.

The regulation of BTEB1 and, for comparison, Sp1 gene expression by E and P, was studied in primary cultures of glandular epithelial (GE) cells isolated from early pregnant pig uterine endometrium in Chapter 3. RT-PCR was used to measure the mRNA abundance of a number of pregnancy-associated genes after optional conditions were determined for each of these reactions. GE cells isolated from D11 and D14 pregnant pig uterine endometrium were treated with different doses of E and/or P after pre-incubation of these cells in medium containing charcoal-stripped fetal bovine serum. These cells were shown to respond to E and P by their increased levels of PR mRNA and uteroferrin (UF) mRNA, respectively. Interestingly, GE cells isolated from D11 or D14 pregnant pigs had distinct dose-dependent responses to hormone treatments. PR mRNA abundance

increased in D11 GE cells only after treatment with 100nM, but was elevated in D14 GE cells upon administration of a lower concentration of E. UF mRNA abundance was not affected by P at a range of concentration in D14 GE cells but was moderately increased ($p=0.09$) in D11 GE cells by treatment with 100nM P. These results suggest that the *in vivo* steroidal environment of the uterine endometrial GE cells influenced their subsequent responses to E and/or P treatments *in vitro*. BTEB1 and Sp1 mRNA abundances were measured in E- and/or P-treated GE cells. BTEB1 mRNA levels increased in D14 GE cells upon treatments with 10nM and 100nM E, while those of Sp1 increased only in these cells at the higher E concentration. There was no change in the mRNA abundance of BTEB1 and Sp1 in D11 GE cells treated with E, while P treatments had no effect, irrespective of pregnancy day. GE cells that were freshly isolated from D11 pregnant pig endometrium exhibit higher BTEB1 and Sp1 mRNA levels than comparable cells from D14 of pregnancy. Thus, the increase in BTEB1 and Sp1 mRNA levels induced by E in uterine endometrial GE cells is quite modest and sensitive to the basal levels *in vivo*. Western blot was used to measure BTEB1 and Sp1 protein abundance upon E and/or P (1 h or 24 h) treatments in GE cells isolated from pregnancy endometrium at D10, a period of low E background *in vivo*. Interestingly, there was no detectable change in either BTEB1 or Sp1 protein abundance with any of the steroid treatments. Two possibilities may account for these observations: one, BTEB1 gene is post-transcriptionally regulated (Imataka et al., 1994; Wang et al., 1997). Although BTEB1 mRNA abundance increases when cells were treated with E, regulation at the level of translation may account for the lack of direct correlation between mRNA and protein levels. However, Sp1 is usually considered as a ubiquitously expressed

transcription factor, and it seems to be transcriptionally regulated since the concentration of the Sp1 protein changes in parallel with that of its mRNA (Imataka et al., 1992). A novel observation here is the difference in E responses observed between Sp1 mRNA and protein. A second possibility is that the variability in response is due to animal variance. Thus, even at the same pregnancy stage, animals can have variations in their hormone and receptor levels. The increases in mRNA abundance of BTEB1 and Sp1 in D14 GE cells treated with E were quite modest, and possibly, limitation in the sensitivity of the Western blot protocol may have prevented any slight changes in protein abundance. Overall, BTEB1 and Sp1 gene expression are not dramatically altered by the pregnancy hormones, E and P.

Several previous studies have indicated that Sp1 modulates the transcriptional response of a number of genes to steroid hormones through protein-protein interactions between Sp1 and nuclear receptors (Owen et al., 1998; Qin et al., 1999; Husmann et al., 2000; Lu et al., 2000; Petz and Nardulli, 2000). The relationship between BTEB1 and PR isoforms A and B respectively was investigated and elucidated in Chapter 4. The sequences of a progesterone-responsive gene promoter, UF (-1143), and those of two non-progesterone-responsive gene promoters, IGFBP-2 (-1397), IGFBP-2 (-305) were analyzed with respect to presence of GC-box and progesterone-response element (PRE), respectively. Both UF and IGFBP-2 promoter (-1397) have four or three GC boxes, but only the UF promoter has functional PRE. These three promoter constructs were separately co-transiently transfected with PR-B expression construct in the presence or absence of P into the uterine endometrial adenocarcinoma (Hec-1-A) sublines exhibiting higher or lower BTEB1 protein content. Results showed that all three promoters had

higher transactivation function in lines containing higher BTEB1 levels than in those with lower BTEB1 content. This indicated that BTEB1 could transactivate gene promoters by binding to GC-enriched sequences. When the PR-B expression construct was present, UF promoter activity increased 4.5 fold over basal activity in 10nM as well as 100nM P in the subline with greater BTEB1 level; by contrast, promoter activity only increased slightly in the subline with lower BTEB1 content in the presence of P. However, this difference in P-dependent promoter activities as a function of cellular BTEB1 content was not observed using IGFBP-2 promoter constructs. These results indicate that the relative amounts of BTEB1 in P-responsive cells can influence the sensitivity of the target gene promoter to transactivation by ligand-bound PR-B. However, P can not influence the transactivation of BTEB1 through PR-B on BTEB1-sensitive, but P-independent promoter activity. Another transfection experiment was used to provide further support to these findings. The MMTV promoter, which contains a functional PRE but no GC box, was used as a reporter construct, and co-transfected with BTEB1 and/or PR-B with and without P. The results showed that BTEB1 and PR-B expression constructs together in the presence of ligand induced greater MMTV promoter activity than any of alone. Parallel studies to determine the BTEB1 effect on PR-A function in progesterone-responsive gene promoters indicated that BTEB1 may not directly interact with PR-A, but may inhibit the transactivity of PR-A and PR-B hetero-dimer. PR-A is known to recruit transcriptional repressors, BTEB1 may amplify the repressor activity of PR-A by interacting with the PR-A/PR-B heterodimer. The study was expanded to another Sp/Krüppel like family member, BTEB3, which is the most similar to BTEB1 in primary sequence. Co-transfection experiments using UF gene promoter and BTEB1,

BTEB3 and PR-B expression constructs in Hec-1-A cells were carried out to determine if BTEB1 and BTEB3 function similarly. The results indicate that a similar interaction between BTEB3 and PR-B occurs as that observed between BTEB1 and PR-B, in a ligand-dependent manner. Taken together, these studies suggest common interactions between Sp/Krüppel-like family members and PR-B.

To further investigate the mechanism behind the effect of BTEB1 on uterine endometrial epithelial cell proliferation (Zhang et al., 2001; Simmen et al., 2002), a putative BTEB1 down-stream gene, namely that encoding secretory leukocyte protease inhibitor (SLPI), was studied in Chapter 5. In a previous study (Zhang et al., 2001), SLPI gene expression was demonstrated to be regulated by BTEB1, but not BTEB2 as determined by measurements of SLPI mRNA abundance in cells containing different levels of BTEB1 as well as the examination of the effect of BTEB1 and BTEB2 on SLPI promoter activity. Further, since most epithelial cells contain a high level of SLPI, it is difficult to study the effect of SLPI on cell proliferation by the usual procedures of *in vitro* addition of SLPI to cell cultures. Thus, an "*in vitro* knock-out" model was established by stably transfecting SLPI-antisense cDNA expression constructs into Ishikawa cells, a highly differentiated uterine endometrial adenocarcinoma cell line. Through RT-PCR, Northern blot, and Western blot analyses, several sublines (e.g. 8As, 11As, and 18As), which express lower levels of SLPI mRNA and protein (8As<11As<18As) relative to parental Ishikawa cells or Ishikawa cells stably transfected with the empty expression vectors, were obtained. Compared to the parental Ishikawa, and control sublines (13pIND), As lines were found to have lower growth rates and lower DNA syntheses rates (as measured by cell counts and [^{3}H] thymidine incorporation).

These results indicate that SLPI is a likely transducer of a BTEB1 pathway involved in the regulation of uterine endometrial epithelial cell proliferation. Subsequent studies focused on the mechanism behind SLPI modulation of cell proliferation. The expression of several cell cycle related genes was investigated in parental cells and sublines expressing different levels of SLPI. Interestingly, cyclin D1 expression at the levels of mRNA and protein was positively correlated with SLPI level in contrast to that of transforming growth factor (TGF)-β1 expression that showed an inverse relationship with cellular SLPI content. Differential display RT-PCR (dd-PCR) and IGF-I ligand blot analysis using sublines containing distinct SLPI levels identified lysyl oxidase (LOX) and IGFBP-3 as potential SLPI gene targets, since their expression negatively correlated with SLPI abundance in epithelial cells. Since cyclin D1 is an important molecule in the transformation of cells from G1 to S phase of the cell cycle, and numerous studies have demonstrated that TGF-β1, LOX and IGFBP-3 are cell growth inhibitors, the results in this study established a possible mechanism underlying SLPI's role as a growth factor. When SLPI was introduced to control (13pIND) and antisense (11As, and 8As) sublines, either through addition of exogenous recombinant SLPI protein into cell medium, or through transfection of an SLPI expression construct into 8As cells, only cyclin D1 mRNA increased, while those for TGF-β1, LOX, IGFBP-3 did not show significant changes. The effect of SLPI on the cyclin D1 promoter activity was studied when normal Ishikawa cells were cotransfected with SLPI expression construct and cyclin D1 promoter construct. SLPI increased cyclin D1 promoter activity at a low concentration but inhibited it at a higher concentration. These results raise the possibility that SLPI may directly regulate cyclin D1 gene expression in an autocrine manner by binding its

putative receptor, which has been identified as scramblase in one study (Tseng and Tseng, 2000); SLPI regulation of the expression of other genes may occur indirectly. Addition experiments indicated that SLPI and IGFBP-3 gene expression were suppressed by TGF-β1, while it increased its own expression and that of LOX. Cyclin D1 gene expression was not altered by TGF-β1. These results provided insights into the complex relationships between these molecules, and indicated that TGF-β1 may suppress SLPI gene expression to further inhibit cell proliferation, and that the inhibitory effect of SLPI on LOX may occur through suppression of TGF-β1 gene expression.

In summary, the studies in this dissertation have provided new insights on the mechanisms by which BTEB1 regulates uterine endometrial epithelial cell differentiation and proliferation. Although the cellular levels of BTEB1 protein are not robustly altered by steroid hormones, E and P, this nuclear protein can significantly affect progesterone-responsive gene expression through its interaction with PR, which is isoform-specific. BTEB1 also can regulate endometrial cell proliferation through its distinct effects on gene expression of SLPI, which subsequently alters the expression of positively- and negatively-acting growth mediators.

Although the studies in this dissertation have yielded an interesting outlook on the important roles of BTEB1 on success implantation and pregnancy, there are further studies to be performed to more thoroughly understand the complicated process of pregnancy. These further studies may include: 1) the regulation of the temporal- and spatial-pattern of expression of BTEB1 in uterine endometrium by autocrine/paracrine-acting cytokines and growth factors. 2) elucidation of the direct effects of BTEB1 on uterine endometrial epithelial cell proliferation and differentiation through identification

of additional progesterone-responsive genes whose transactivation or transrepression also involve BTEB1 regulation. 3) *in vivo* studies including the BTEB1, PRs, alone or in combination, "knock-out" models 4) detailed investigations of the interaction domains between BTEB1 and PRs may yield more insights about the interactions between KLF family members and nuclear receptors. 5) identification of the SLPI receptor and/or the signaling pathway that SLPI uses as a growth factor. 6) further identification of the interactive signaling pathways that are involved in the modulation of uterine endometrial epithelial cell proliferation by SLPI, pathways involving cyclin D1 (ras/MAPK and NF-κB), TGF-β signaling system (Smads), and LOX.

Investigation of the role of BTEB1 on uterine endometrial cell proliferation and differentiation is important and will benefit animal agriculture and human health. P is an essential steroid hormone for successful pregnancy either in animals and the human. The demonstration of the molecular effects of BTEB1 on PR/P activity allows for a more thorough and complete understanding of P action and provides possible direction for further therapies to avoid embryo losses and unexpected pregnancy termination. In addition, the discovery of the regulation of cell proliferation by BTEB1 involving SLPI and several growth-related factors gives novel insights applicable to embryo implantation and pregnancy, tumorigenesis, inflammatory processes, and other important clinical conditions.

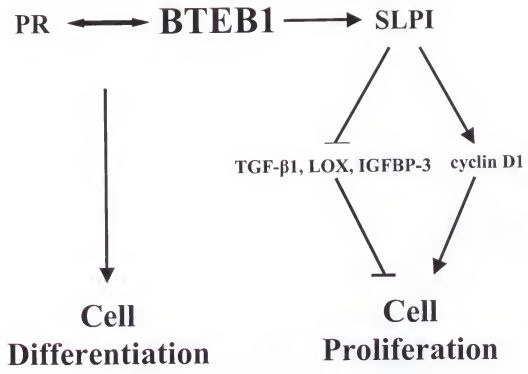


Figure 6-1. A model for the role of BTEB1 in uterine endometrial epithelial cell differentiation and proliferation. BTEB1 affects progesterone-responsive gene expression, which is indicated as cell differentiation signals, through interaction with progesterone receptor. One possible pathway by which BTEB1 modulates epithelial cell proliferation is through its control of SLPI gene expression, which in turn modulates cyclin D1 gene expression (increase) and TGF-b1, LOX and IGFBP-3 gene expression (decrease).

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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